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13. ABSTRACT *(Maximum 200 words)*

The main purpose of my research proposal is to understand the regulation of Wingless signaling by heparan sulfate proteoglycan (HSPG). I have previously cloned the *Drosophila sugarless (sgl)* and *sulfateless (sfl)* genes that encode enzymes required for the biosynthesis of heparan sulfate glycosaminoglycans. In this report, I have conducted further characterization of *sfl* gene, which is required for Wingless (Wg) signaling in both embryonic and wing imaginal disc development. I have also identified Dally, a GPI-linked Glypican, as the HSPG molecule involved in Wg signaling. Loss of dally activity, both in the embryo and wing imaginal discs, generates phenotypes reminiscent of loss of Wg activity. Our results indicated that Dally may act as a co-receptor for Wg, and that Dally, together with Wg receptor *Drosophila Frizzled 2*, modulates both short- and long-range activities of Wg. Furthermore, in addition to Wg signaling, I have also demonstrated that *sgl* and *sfl* mutant embryos have phenotypes similar to those lacking the functions of two Drosophila fibroblast growth factor receptors. My results provide the first genetic evidence that heparan sulfate glycosaminoglycans are essential for fibroblast growth factor receptor signaling.

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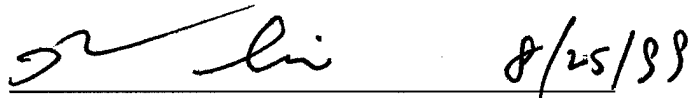

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Introduction

Heparan sulfate proteoglycans (HSPGs) is one of the major classes of cell surface molecules that play critical roles in various cellular processes such as cell adhesion, neurite outgrowth, angiogenesis, tumorigenesis. The glycosyl phosphatidylinositol (GPI)-linked Glypicans and the transmembrane Syndecans represent the two major cell surface HSPGs. *Drosophila* Wingless (wg) encodes a homolog of vertebrate Wnt-1 protein that is implicated in breast cancer etiology. Biochemical studies have shown that both Wg and its mouse homolog Wnt-1 are high-affinity Heparin-binding proteins. I have recently reported that in the absence of *sugarless (sgl)* and *sulfateless*, which encode enzymes involved in the biosynthesis of heparan sulfate proteoglycan, Wg signaling is defective. These results suggest that HSPG(s) play a key role(s) in Wg signaling. The goals of this fellowship study are to utilize powerful *Drosophila* genetics to study further the mechanism(s) of how HSPGs regulate Wg signaling. Specifically, I would like to investigate that 1. how Wg signaling is regulated in the absence of Sfl activity. 2. Which HSPG protein core is involved in Wg signaling. 3. How HSPG cooperates with Wg and its receptor to modulate Wg signaling. 4. What is the specificity of HSPG in Wg and other growth factor signaling. Towards these goals, I have conducted a number of experiments to investigate the regulation of Wg signaling in various tissue of *sfl* mutant and to identify the HSPG that is involved in Wg signaling. I have also conducted several experiments to investigate the function of HSPG in signal transduction of FGF, which is one of the essential growth factors implicated in the development of breast cancer.

Body of Annual Summary.

1. Sfl is involved in Wg signaling

1.1. *Sfl is involved in Wg signaling during embryonic development.*

sfl mutant was identified in a genetic screen to characterize the maternal effects of zygotic lethal mutations. I have previously reported the cloning of *sfl* and preliminary characterization of *sfl* mutant. *sfl* encoded a *Drosophila* homolog of vertebrate HS/Heparin N-deacetylase/N-sulfotransferase (NDST). The cuticle phenotype of *sfl* null embryos is reminiscent of the phenotypes exhibited by mutations in *wg*, suggesting that Sfl is required for Wg signaling. To investigate further the role of Sfl in Wg signaling, I examined the development of several other embryonic tissues that require Wg activity. These include the development of the stomatogastric nervous system (SNS) and the second midgut construction. Examination of *sfl* null embryos revealed that the SNS invaginations do not occur properly and resemble those found in *wg* mutant. Further, in *sfl* null embryos, the second midgut constriction does not form as is also observed in *wg* mutants. Together, these results implicate a requirement for Sfl activity in Wg signaling during embryonic development.

1.2. *Sfl is required for both short- and long-range activities of Wg*

To further substantiate a requirement for Sfl activity in Wg signaling, we analyzed the effect of *sfl* mutations during wing imaginal disc development. Wg is required for D/V

patterning and acts as a short-range inducer to activate the expression of several genes such as neuralized (neu) at the wing margin. Wg also functions as a morphogen to directly activate the transcription of several target genes including dll in a concentration-dependent manner. In *sfl* mutant wing discs, the expression of neu is abolished, suggesting that Sfl is required for short-range Wg activity. Further, the expression of Dll is strikingly reduced in *sfl* mutant discs reflecting a role of Sfl in long-range Wg signaling. Consistent with these results, clones of *sfl* mutant cells induced at the first instar stage generate severe wing margin defects, a phenotype also seen with *wg* mutant clones. Thus, our results strongly argue that Sfl is required for both short- and long-range activities of Wg in the wing imaginal disc.

2. Dally, a member of the glypican family of HSPG, is involved in Wg signaling.

2.1. *dally is a new segment polarity gene and is required for Wg signaling in embryonic development.*

The analysis of *sfl* indicates a requirement for HS GAGs, but not other classes of GAGs in proper Wg signaling. Since HS GAGs are attached to various protein cores to form different HSPGs, we searched for candidate genes that could encode the protein core of the HSPG. The *Drosophila* glypican homolog *dally* appeared as an excellent candidate because a previous analysis has reported that flies homozygous for hypomorphic dally alleles exhibit some wing margin defects, a phenotype reminiscent of partial loss of wg activity.

As a first step in examining the role of dally in Wg signaling, we determined the expression of dally mRNAs in embryos by in situ hybridization. We found that *dally* is expressed both maternally and zygotically. At early stages *dally* transcripts are uniformally expressed. However, at stage 8 they are enriched in a segmental repeated pattern. In stage 8 embryos, dally transcripts are expressed in three to four cells anterior to wg-expressing cells. Interestingly, double staining for Dfz2 mRNA and wg-lacZ reveals that the 2 to 3-cell wide band of dally-expressing cells anterior to wg-expressing cells also express Dfz2, a Wg receptor, suggesting a possible role for dally in Wg signaling.

To demonstrate that Dally is required for Wg signal transduction, we examined the cuticle phenotype of *dally* mutant embryos. Hypomorphic *dally* mutant embryos exhibit poorly penetrant cuticle segment polarity defects resembling a partial defect in Wg signaling. However, the segment polarity defect associated with *dally* mutant can be significantly enhanced by removal of one copy of *sfl* in the mother or one copy of *wg* in the embryo. These genetic dosage interactions are consistent with a role of Dally in Wg signaling.

Because all available *dally* mutants are weak alleles, we used double-stranded RNA (dsRNA) interference as a method to abrogate the activity of the endogenous *dally* gene. We injected dsRNAs corresponding to the entire coding region of *dally* into wild type embryos. Embryos injected with the *dally* dsRNAs exhibit severe segment polarity cuticle defects, similar to those injected with *wg* or Wg receptor fz + Dfz2 dsRNAs. This result, together with the genetic interaction observed between *sfl* and *wg* strongly argues that *dally* is a new segment polarity gene and that it is required at least for Wg signaling in the embryo.

2.2 *Dally is required for Wg signaling in Wg patterning.*

To further examine the role of *dally* in Wg signaling, we analyzed the function of *dally* during wing imaginal disc development. we observed that only 3% of homozygous *dally* animals that carry the weak alleles *dally* p2 exhibit wing margin defects. This frequency can

be increased by 2- to 3- fold and wing margin defects are more severe when a single copy of *wg* is removed. The enhancement of the wing defects of dally mutant by a reduction in Wg activity suggests that Dally plays a role in Wg signaling at the wing margin. To determine whether Dally cooperates with the Wg receptor Dfz2 in wing patterning, we tested whether *dally* mutations can enhance a loss of function Dfz2 phenotype. Ectopic expression of a dominant negative form of Dfz2 (Dfz2N), that encodes only the first extra-cellular domain and the first transmembrane domain, has been shown to block Wg signaling, probably by binding to Wg in a non productive manner. When Dfz2N is expressed ectopically using the Gal4 line C96, which drives expression in the presumptive wing margin, flies develop partial margin defects. However, this phenotype is dramatically enhanced in homozygous *dally* mutants, suggesting that Dally potentiates Wg signaling. More importantly, ectopic expression of a gain of function Arm protein can fully rescue the wing defects, suggesting that the enhanced wing margin defects by *dally* mutant is specific to Wg signaling and that Dally acts upstream of Arm. These genetic interactions are consistent with a role of Dally in Wg signaling and suggest that Dally may act together with Dfz2 in Wg reception.

2.3 *Dally cooperates with Dfz2 to transduce wingless signaling*

If Dally acts together with Dfz2 to transduce Wg signaling, Dally may also be required for the other functions of Dfz2 in Wg signaling. In the wing blade, Dfz2 is involved in shaping the gradient of Wg distribution and determining the response of cells to Wg. Uniform overexpression of Dfz2 in the wing pouch leads to ectopic bristle formation in the wing blade, most likely reflecting the activation of Wg signaling above its normal level. Ectopic expression of Dfz2 driven by the Gal4 line 69B resulted in wings with ectopic bristles. In a *dally* mutant background, the formation of ectopic bristles was drastically reduced suggesting that a mutation in *dally* blocks the activity of Dfz2. This result suggests that Dally cooperates with Dfz2 to transduce Wg signaling.

3. Genetic evidence that HSPG is required for FGF receptor signaling

Althrough the study about the role of HSPG in FGF signaling is not the major goal of my initial proposal, in order to determine whether HSPGs regulate other growth factor signaling other than Wg, I have also conducted several experiments to investigate if FGF signaling is defective in the absence of Sfl and Sgl activity. This part of study is not only important to understand the specificity of HSPG in Wg signaling, but itself is also very important since FGF is another key growth factor implicated in the development of breast cancer.

Two FGFRs, Heartless (Htl) and Breathless (Btl), have been characterized in *Drosophila*. Genetic analyses have established that each of these receptors has distinct expression patterns and developmental functions during embryogenesis. Htl is expressed uniformly in the early embryonic mesoderm where it is required for the normal dorsolateral migration of mesodermal cells following gastrulation □Btl is expressed in the tracheal system as well as in a subset of cells in the CNS midline. Htl and Btl are required for mesodermal cell and tracheal cell migration respectively.

3.1. *sfl and sgl mutants phenocopy cell migration defects associated with loss of Htl or Btl function*

In *htl* mutant embryos, gastrulation is normal but mesoderm migration fails to occur properly, resulting in an irregular dorsal margin of Twi-positive cells and a relative accumulation of

these cells in ventral and lateral positions. A similar phenotype occurs in *sfl* and *sgl* null embryos, suggesting that in the absence of *sgl* and *sfl* activity, Htl FGF receptor signaling is defective. We have found that a constitutively active form of Htl can bypass the requirement of sfl and sgl for mesoderm migration, which further supports that Htl FGF receptor signaling is abolished in *sfl* and *sgl* mutants. These results concluded that Htl signaling requires HSPG. We have also conducted a genetic interaction experiment with weak allele of *htl* and demonstrated that the interactions between *htl* and both *sfl* and *sgl* are highly significant, supporting our conclusion that HSPG is required for Htl signaling

Given the genetic evidence that Sfl and Sgl are required for Htl FGFR signaling in the mesoderm, we next determined whether HS GAG biosynthesis is also involved in signaling by Btl during trachea development. In either *sfl* or *sgl* zygotic mutants, tracheal branch formation is incomplete.The relatively weak zygotic *sfl* and *sgl* tracheal phenotypes are most likely due to partial rescue by the maternal expression of these genes. In the null *sgl* and *sfl* mutant embryos, tracheal cell migration is completely abolished. In summary, these findings implicate that Sfl- and Sgl-dependent HS GAG biosynthesis in signaling by the Btl FGFR.

3.2 Htl- and Btl-dependent MAPK activation depends on Sfl and Sgl

Htl and Btl are RTKs that transduce their intracellular signals by the conserved Ras/MAPK cascade. As a result, RTK activity can be visualized in developing tissues with an antibody specific for the diphosphorylated, activated form of MAPK. In both *sgl* and *sfl* null mutant embryos, either Htl or Btl associated MAPK activities are abolished as observed in *htl* and *btl* null mutant embryos. in a control experiment, MAPK activity associated with Htl and Btl is not changed in wg mutant embryos. These in situ patterns of MAPK activation provide direct evidence that Sfl and Sgl are required for signaling by the two Drosophila FGFRs, Htl and Btl, independent of the requirement for HS GAGs in Wg function

Appendix:

Enclosed are two papers that described in details about all the work summarized in this annual report.

1. Xinhua Lin and Norbert Perrimon (1999). Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signaling. **Nature** Vol. 400, 281-283.

2. Xinhua Lin, Eugene M. Buff, Norbert Perrimon and Alan Michelson (1999). Heparan sulfate Proteoglycan are essential for FGF receptor signaling during Drosophila embryonic development. **Development in press**

Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling

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The *Drosophila wingless* gene (*wg*) encodes a protein of the Wnt family and is a critical regulator in many developmental processes[1]. Biochemical studies have indicated that heparan sulphate proteoglycans, consisting of a protein core to which heparan sulphate glycosaminoglycans are attached[2], are important for Wg function[3]. Here we show that, consistent with these findings, the *Drosophila* gene *sulfateless* (*sfl*), which encodes a homologue of vertebrate heparan sulphate *N*-deacetylase/*N*-sulphotransferase (an enzyme needed for the modification of heparan sulphate) is essential for Wg signalling. We have identified the product of *division abnormally delayed* (*dally*), a glycosyl-phosphatidyl inositol (GPI)-linked glypican, as a heparan sulphate proteoglycan molecule involved in Wg signalling. Our results indicate that Dally may act as a co-receptor for Wg, and that Dally, together with *Drosophila* Frizzled 2, modulates both short- and long-range activities of Wg.

Wg signalling is defective in *sugarless* (*sgl*) mutants[4]. *sgl* encodes a *Drosophila* homologue of uridine diphosphoglucose dehydrogenase that is required for the formation of glucuronic acid. Because glucuronic acid is required for the formation of heparan sulphate, chondroitin sulphate and dermatan sulphate, it is unclear which classes of proteoglycans are involved in Wg signalling. In the genetic screen[5] that led to the isolation of *sgl*, we also isolated mutations in a second locus *sulfateless* (*sfl*), that showed a similar segment-polarity cuticle phenotype (Fig. 1b). In *sfl* null embryos, the expression patterns of *wg* and *engrailed* (*en*) are reminiscent of those observed in either *wg* or *hedgehog* (*hh*) null mutants (not shown). To investigate further whether Sfl activity is required in Wg signalling, we analysed the effect of *sfl* mutations on the development of stomatogastric nervous system (SNS) and the second midgut constriction, both of which require Wg but not Hh activity[6]. In *sfl* null embryos, the development of these Wg-mediated processes is perturbed (Fig. 1e, g). Consistent with a role for Sfl in Wg signalling, Wg-dependent processes in the wing imaginal disc also require Sfl activity. Wg is required for dorso–ventral patterning and acts over a short range to control the expression of *neuralized* (*neu*) at the wing margin[1] and in the long range to activate the expression of *distaless* (*dll*)[7,8]. In *sfl*-mutant wing discs, the expression of *neu* is abolished (Fig. 1i), and Dll expression is also markedly reduced (Fig. 1k). Our results indicate that Sfl activity is necessary for Wg signalling during both embryonic and wing-disc development.

A complementary DNA encoding the product of *sfl* was isolated (Fig. 2). A search of the protein sequence databases revealed that the putative protein deduced from the *sfl* cDNA is homologous with heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST)[9], which is required specifically for the modification of heparan sulphate glycosaminoglycans (GAGs) but not chondroitin sulphate and dermatan sulphate GAGs. Together, these results provide genetic evidence that heparan sulphate proteoglycans (HSPGs) are involved in Wg signalling and that HSPGs have non-redundant roles with other classes of proteoglycan in the context of Wg signalling.

Heparan sulphate GAGs are attached to various protein cores to form different HSPGs. Because *Drosophila* frizzled 2 (Dfz2) appears as a distinct band on western blots (S. Cumberledge, personal communication; M. Zeidler and N.P., unpublished), not as the smear that is characteristic of proteoglycans[2], it is unlikely that the receptors for Wg/Wnt encoded by members of the Frizzled (Fz) family are heparan-sulphate-modified proteins. The *Drosophila glypican* homologue *dally* appeared to be an excellent candidate because flies homozygous for hypomorphic *dally* alleles exhibit some wing-margin defects[10], a phenotype similar to partial loss of *wg* activity. To examine the role of *dally* in Wg signalling, we first determined the expression of *dally* messenger RNAs in embryos by *in situ* hybridization (Fig. 3). At early stages *dally* transcripts are uniformly expressed; however, at stage 8, *dally* transcripts are enriched in a segmental repeated pattern in three to four cells anterior to *wg*-expressing cells. Double staining for *Dfz2* mRNA and wg–lacZ shows that the 2–3-cell-wide band of *dally*-expressing cells anterior to *wg*-expressing cells also express *Dfz2*[11] (not shown), indicating that *dally* may be involved in Wg signalling.

Next, we examined the cuticle phenotype of *dally* mutant

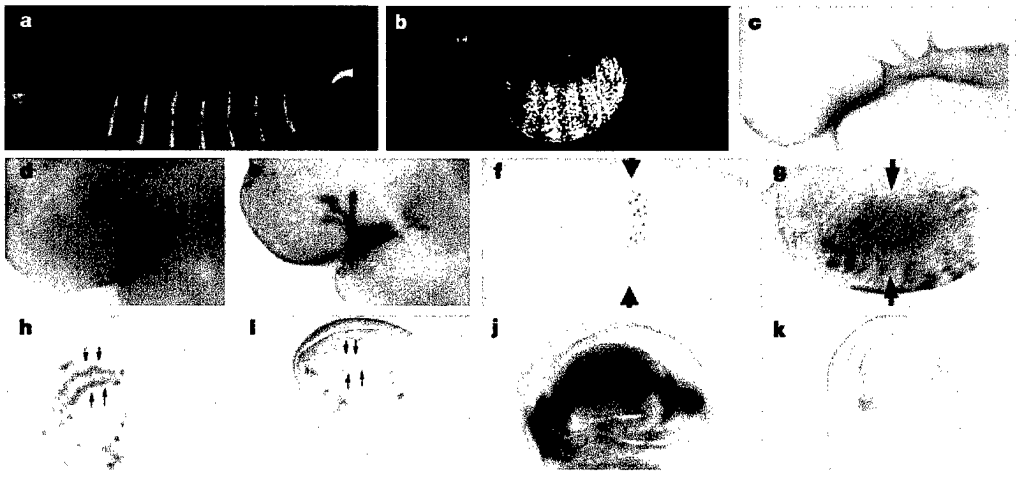

Figure 1 *sfl* is required for Wg signalling. **a**, **b**, Cuticle phenotypes of wild-type (WT) (**a**) and *sfl* (**b**) embryos. **c**–**e**, SNS phenotypes stained by anti-Crumbs antibody in stage 10 embryos of WT (**c**), *wg* (**d**), *sfl* (**e**). As observed for *sgl*[6], the SNS phenotype is similar, although slightly weaker, than in the *wg* mutant (ref. 6). **f**, **g**, The expression of Labial (Lab) in WT (**f**) and *sfl* (**g**) embryos at stage 15; Lab staining, marking the position where the second midgut (arrows) is absent in an *sfl* embryo (**g**). In the wing disc of WT third-instar larvae (**h**), *neuralized* (*neu*)-expressing sensory mother cells visualized using the A101 enhancer trap are found in two rows (arrows), and are missing in $sfl^{(3)03844}/sfl^{9B4}$ wing disc derived from *sfl* homozygous mutant animals derived from heterozygous mothers (**i**). In WT wing disc (**j**), Dll forms a gradient with its highest level of expression at the dorso-ventral boundary, and is greatly reduced in the $sfl^{(3)03844}/sfl^{9B4}$ disc (**k**).

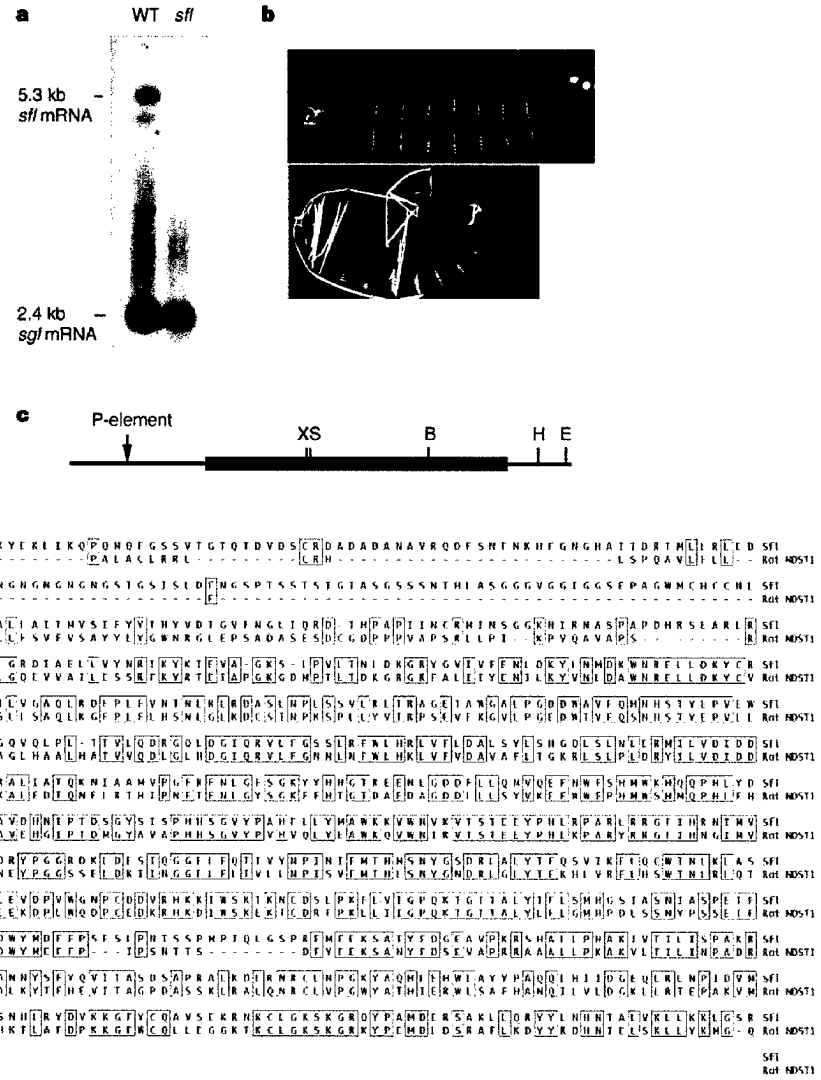

Figure 2 *sfl* encodes heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST). **a**, Northern blot analysis of *sfl* RNA from 0–1.5 h WT and *sfl* mutant embryos. The blot was probed with *sfl* cDNA and *sgl* cDNA[6]. *sgl* was used as an internal control. 5.3 and 2.4 kilobase (kb) mRNAs correspond to the *sfl* and *sgl* transcripts, respectively. **b**, Rescue of *sfl* maternal-effect phenotypes by RNA injection. Top, cuticle phenotype of a paternally rescued *sfl* embryo marked with a *trachealess* (*trh*) mutation that exhibits the defective posterior spiracles[6]. Bottom, cuticle phenotype of *sfl* null embryos derived from GLCs injected with RNA transcribed from the *sfl* full-length cDNA. Of 700 injected embryos derived from females with *trh* GLCs, 120 *sfl* mutant embryos (*trh sfl/sfl*) developed scorable cuticle structures, and 15% of them showed evidence of rescue. **c**, Restriction map of the *sfl* cDNA. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sst*I; X, *Xho*I. The P-element *l(3)03844* is inserted at base 576 of the *sfl* cDNA (686 base pairs (bp) upstream of a putative ATG start codon). The open reading frame encoding Sfl is shown by the thick line. **d**, Putative amino-acid sequence of Sfl protein and comparison with rat NDST1. Identical residues are boxed. The overall identity between Sfl and Rat NDST1 is 51%.

embryos. These mutant embryos exhibit poorly penetrant cuticle segment-polarity defects resembling a partial defect in Wg signalling (Fig. 3d). However, these segmentation defects can be significantly enhanced by removal of one copy of *sfl* in the mother or one copy of *wg* in the embryo (Fig. 3e, f). Because all available *dally* mutations are weak alleles, we used double-stranded RNA (dsRNA) interference to block *dally* gene activity[12]. Embryos injected with *dally* dsRNAs corresponding to the entire coding region of *dally* exhibit severe segment-polarity cuticle defects (Fig. 3g, h), similar to those injected with *wg* or *frizzled* (*fz*) + *Dfz2* dsRNAs[12]. This result, together with the genetic interaction observed between *sfl* and *wg*, strongly supports the proposal that *dally* is a new segment-polarity gene and that it is required for Wg signalling in the embryo. Further, we find that Dally, which migrates as a smear in wild-type extracts, migrates as sharp bands in the protein extracts of *sfl* mutants (Fig. 4). Similarly, Dally is not modified in *sgl* embryos (S. Selleck, personal communication). These results indicate that Dally is a likely substrate of Sgl and Sfl.

To further examine the role of *dally* in Wg signalling, we analysed the function of *dally* during wing-disc development. Consistent with previous reports[10], we found that only 3% of homozygous *dally* animals exhibit wing-margin defects (Fig. 5a). This frequency can be increased 2–3-fold and wing-margin defects are more severe when one copy of *wg* is removed (Fig. 5b). To determine whether Dally cooperates with the Wg receptor Dfz2 in wing patterning, we tested whether *dally* mutations can enhance a loss-of-function *Dfz2* phenotype. When a dominant-negative form of Dfz2 (Dfz2N)[13] is expressed ectopically using the Gal4 line C96, which drives expression in the presumptive wing margin, flies develop partial margin defects (Fig. 5c). However, this phenotype is enhanced in homozygous *dally* mutants (Fig. 5d), indicating that *dally* may potentiate Wg signalling. Furthermore, ectopic expression of a gain-of-function

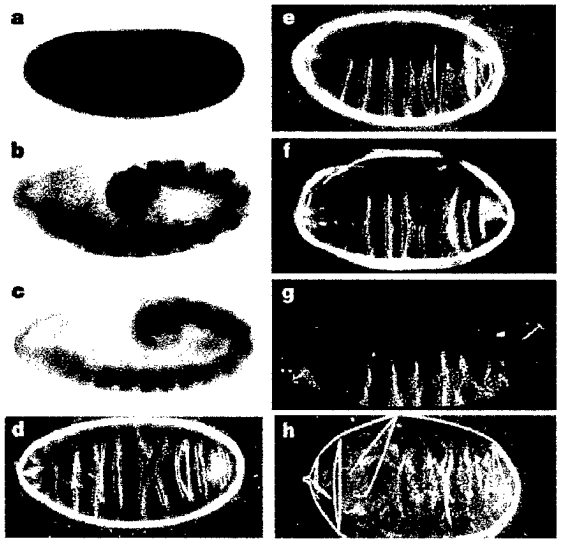

Figure 3 *dally* is a segment-polarity gene. *dally* mRNAs are uniformly distributed in stage 2 embryos (**a**), and are expressed in a segmental repeated pattern at stage 8 (**b**). **c**, *dally* transcripts (blue) are located 3–4 cells anterior to *wg*-expressing cells (brown, *wg–lacZ*). $dally^{P2}$ homozygous embryos derived from females with $dally^{P2}$ or $dally^{\Delta P\text{-}188}$ homozygous GLCs exhibit weak segment-polarity cuticle defects (**d**; 8% penetrance, $n = 760$). More severe defects are detected in $dally^{P2}$ embryos where *sfl* maternal message is eliminated (**e**; 14% penetrance; $n = 780$). Similarly, $dally^{P2}$ homozygous embryos derived from GLCs show a more severe embryonic phenotype if zygotic *wg* is reduced by half (**f**; 16% penetrance, $n = 760$). Embryos injected with *dally* dsRNA develop *wg*-like cuticle defects (**g**, **h**). 48% of the injected embryos ($n = 127$) exhibit defects. Embryos injected with buffer exhibit no cuticle defects ($n = 150$).

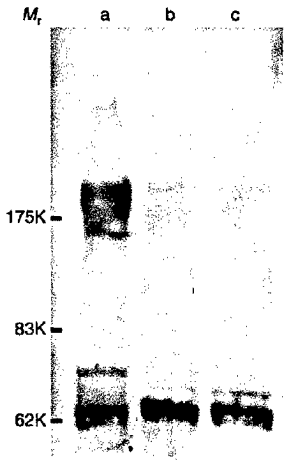

Figure 4 Heparan sulphate GAG modification of Dally in *sfl* mutants. Total proteins from third-instar larvae were analysed by SDS–PAGE followed by western blotting with anti-Dally antibody. In WT larvae (a), Dally migrates as a high relative molecular mass (M_r) smear, characteristic of heparan sulphate-modified Dally, and ~70K unmodified bands. In homozygous $sfl^{l(3)03844}$ (b) or sfl^{9B4} (c) larvae, high M_r heparan sulphate-modified Dally is significantly reduced and sharp bands of unmodified Dally are increased.

Arm protein (Arm^{act}) can fully rescue the wing defects (Fig. 5f), indicating that the enhancement of wing-margin defects in the *dally* mutant is specific to Wg signalling and that Dally acts upstream of Arm. These genetic interactions are consistent with a role for Dally in Wg signalling and indicate that Dally may act with Dfz2 in Wg reception.

If *dally* acts with Dfz2 to transduce Wg signalling, Dally may also

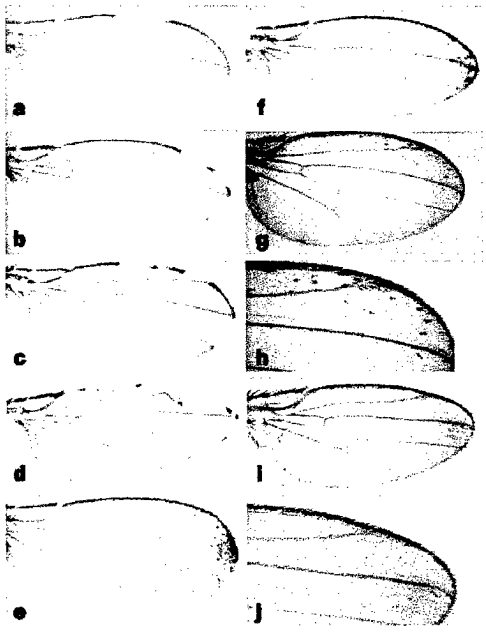

Figure 5 Dally is required for Wg/DFz2 signalling in wing patterning. **a**, 3% of $dally^{P2}$ homozygous flies exhibit a wing notching phenotype ($n = 550$). **b**, Wing phenotype of wg^{IG22}/+; $dally^{P2}/dally^{P2}$ animals is enhanced and shows higher penetrance (8%; $n = 654$). The wing-vein abnormality seen in the *dally* mutant does not seem to involve Wg signalling. **c**, Ectopic expression of Dfz2N at the presumptive wing margin using C96-Gal4 (*UAS-Dfz2N*/+; *C96*/+) is associated with a fully penetrant mild wing-margin defect. **d**, *UAS-Dfz2N*/+; *C96* $dally^{P2}/dally^{P2}$ wing. Decreased *dally* activity strongly enhances the wing defect observed in **c**. **e**, UAS-arm^{act}/+, C96/+ wing. Ectopic expression of arm^{act} results in ectopic bristles on the wing blade. **f**, UAS-arm^{act}/*UAS-Dfz2N*; *C96* $dally^{P2}/dally^{P2}$ wing. Ectopic expression of UAS-arm^{act} fully rescues the margin defect shown in **d**. **g**, **h**, Uniform expression of Dfz2 driven by 69B-Gal4 in *69B-Gal4/UAS-Dfz2* flies leads to wings with ectopic bristles. **i**, **j**, Ectopic bristles are strikingly reduced in the wing of *UAS-Dfz2* $dally^{P2}$/*69BGal4* $dally^{P2}$. **h**, **j**, Higher magnifications of the wings shown in **g** and **i**, respectively.

be required for other functions of Dfz2 in Wg signalling. In the wing blade, Dfz2 is involved in shaping the gradient of Wg distribution and determining the response of cells to Wg[14]. Uniform overexpression of *Dfz2* in the wing pouch leads to ectopic bristle formation in the wing blade, probably reflecting activation of Wg signalling above its normal level. Ectopic expression of *Dfz2* driven by the Gal4 line 69B resulted in wings with ectopic bristles[14] (Fig. 5g, h). In a *dally* mutant background, the formation of ectopic bristles was greatly reduced, indicating that a mutation in *dally* blocks the activity of Dfz2 (Fig. 5i, j).

Our findings indicate that HSPGs have non-redundant roles with other classes of proteoglycan in Wg signalling, and that *dally* encodes a protein core of the HSPGs involved in Wg signalling. There are several possible mechanisms for the function of Dally in Wg signalling. First, Dally could form an active Wg receptor complex with Dfz2. Second, Dally, through its heparan sulphate GAG sequences, could generate a higher-affinity binding site for Wg to Dfz2. Third, as proposed for other co-receptors[15], Dally could limit the free diffusion of Wg by capturing it on the cell surface, thereby increasing its local concentration and the probability that it will interact with less abundant, high-affinity signalling receptors. Biochemical analyses between Dally, Wg and Dfz2 will be required to distinguish between these models. Interestingly, both *Dfz2* and *Fz* encode redundant Wg receptors in the embryo[12]. Thus it is possible that, in addition to having a role in the Wg/Dfz2 interaction, Dally also cooperates with Wg/Fz. Furthermore, Dally regulates the

activity of decapentaplegic (Dpp)[16], a member of the TGF-β superfamily. As we have no evidence for a role for HSPGs in the early function of Dpp in the establishment of dorso–ventral embryonic polarity, the function of Dally may be tissue-specific. Tissue-specific effects of Dally could be generated either through tissue-specific expression of *dally* during development or tissue-specific modification of the heparan sulphate GAG chains linked to the Dally protein core. There is biochemical and genetic evidence to support the model that specific heparan sulphate GAGs decorate the cell surface. In vertebrates, a number of sulphotransferases are differentially expressed in various tissues[17]. In addition, the *Drosophila* gene *pipe*, which is involved in dorso–ventral patterning in the embryo, encodes a putative heparan sulphate 2-*O* sulphotransferase that is expressed in ventral follicle cells[18]. □

Methods

Reagents. The *sfl* alleles are $sfl^{l(3)03844}$ (ref. 5) and sfl^{9B4} (N.P., unpublished). Both $sfl^{l(3)03844}$ and sfl^{9B4} show similar maternal-effect phenotypes. Females with germline clones (GLCs) were generated as described[6]. All the available *dally* alleles are homozygous viable to some extent, with $dally^{P2}$ and $dally^{AP188}$ representing the strongest alleles available[10]. To try to isolate a stronger loss-of-function *dally* allele, we generated a number of new *dally* alleles by P-element excisions. However, none was stronger than the original[10]. Other stocks are: UAS–Dfz2 (ref. 14) and UAS–Dfz2N (ref. 13), UAS–arm^{act} (ref. 19), C96 Gal4 (ref. 20). *dally* cDNA was obtained from S. Selleck[10]. Crumbs, Dll and Lab antibodies were obtained from E. Knust, I. Duncan and T. Kaufman, respectively.

Molecular methods. Molecular characterization of *sfl* and RNA injection were done as described for *sgl*[6]. Western blotting of Dally was performed using a polyclonal Dally antibody, a gift of H. Nakato. The dsRNA synthesis and injection were as described[12].

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1. Siegfried, E. & Perrimon, N. *Drosophila* wingless: a paradigm for the function and mechanism of Wnt signaling. *BioEssays* **16**, 395–404 (1994).
2. David, G. Integral membrane heparan sulfate proteoglycans. *FASEB J.* **7**, 1023–1030 (1993).
3. Reichsman, F., Smith, L. & Cumberledge, S. Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. *J. Cell Biol.* **135**, 819–827 (1996).
4. Cumberledge, S. & Reichsman, F. Glycosaminoglycans and WNTs: just a spoonful of sugar helps the signal go down. *Trends Genet.* **13**, 421–423 (1997).
5. Perrimon, N., Lanjuin, A., Arnold, C. & Noll, E. Zygotic lethal mutations with maternal effect phenotypes in *Drosophila melanogaster*. II. Loci on the second and third chromosomes identified by P-element-induced mutations. *Genetics* **144**, 168–1692 (1996).
6. Haecker, U., Lin, X. & Perrimon, N. The *Drosophila sugarless* gene modulates. Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565–3573 (1997).
7. Neumann, C. J. & Cohen, S. M. Long-range action of Wingless organizes the dorsal–ventral axis of the *Drosophila* wing. *Development* **124**, 871–880 (1997).
8. Zecca, M., Basler, K. & Struhl, G. Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833–844 (1996).
9. Hashimoto, Y., Orellana, A., Gil, G. & Hirschberg, C. B. Molecular cloning and expression of rat liver N-heparan sulfate sulfotransferase. *J. Biol. Chem.* **267**, 15744–15750 (1992).
10. Nakato, H., Futch, T. A. & Selleck, S. B. The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. *Development* **121**, 3687–3702 (1995).
11. Bhanot, P. *et al.* A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature* **382**, 225–230 (1996).
12. Kennerdell, J. R. & Carthew, R. W. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* **95**, 1017–1026 (1998).
13. Zhang, J. & Carthew, R. W. Interactions between Wingless and DFz2 during *Drosophila* wing development. *Development* **125**, 3075–3085 (1998).
14. Cadigan, K. M., Fish, M. P., Rulifson, E. J. & Nusse, R. Wingless repression of *Drosophila* wing development. *Development* **125**, 3075–3085 (1998).
15. Schlessinger, J., Lax, I. & Lemmon, M. Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell* **83**, 357–360 (1995).
16. Jackson, S. M. *et al.* dally, a Drosophila glypican, controls cellular responses to the TGF-β-related morphogen, Dpp. *Development* **124**, 4113–4120 (1997).
17. Bullock, S. L., Fletcher, J. M., Beddington, R. S. & Wilson, V. A. Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev.* **12**, 1894–1906 (1998).
18. Sen, J., Goltz, J. S., Stevens, L. & Stein, D. Spatially restricted expression of pipe in the *Drosophila* egg chamber defines embryonic dorsal–ventral polarity. *Cell* **95**, 471–481 (1998).
19. van de Wetering, M. *et al.* Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell* **88**, 789–799 (1997).
20. Gustafson, K. & Boulianne, G. L. Distinct expression patterns detected within individual tissues by the GAL4 enhancer trap technique. *Genome* **39**, 174–182 (1996).

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The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins

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The regulation of gene expression depends critically upon chromatin structure[1]. Transcription of protein-coding genes can be reconstituted on naked DNA with only the general transcription factors and RNA polymerase II (ref. 2). This minimal system cannot transcribe DNA packaged into chromatin, indicating that accessory factors may facilitate access to DNA. Two classes of accessory factor, ATP-dependent chromatin-remodelling enzymes[3] and histone acetyltransferases[4], facilitate transcription initiation from chromatin templates. FACT (for facilitates chromatin transcription) is a chromatin-specific elongation factor required for transcription of chromatin templates *in vitro*[5,6]. Here we show that FACT comprises a new human homologue of the *Saccharomyces cerevisiae* Spt16/Cdc68 protein and the high-mobility group-1-like protein structure-specific recognition protein-1. Yeast *SPT16/CDC68* is an essential gene that has been

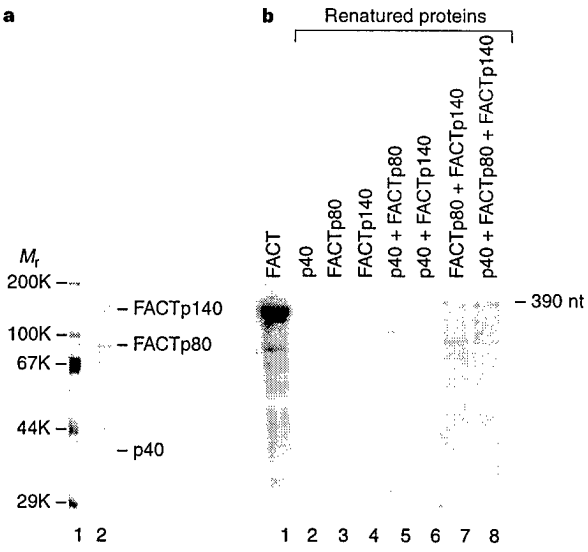

Figure 1 Recovery of FACT activity from renatured p140 and p80 subunits. **a**, Silver-stained gel (phosphocellulose column, fraction 9) showing proteins used in FACT renaturation experiment. The positions of FACTp140, FACTp80, the p40 protein and protein relative molecular mass (M_r) markers are indicated. **b**, Mixtures of renatured polypeptides assayed for FACT activity. Polypeptides were renatured alone and in all possible combinations and were used in transcription reactions on remodelled chromatin templates containing Gal4-VP16 and a reconstituted transcription system (lanes 2–8). Purified FACT was used in lane 1. FACT activity was measured by the appearance of long RNA molecules. The position of the full-length, 390-nucleotide RNA product is indicated.

Heparan Sulfate Proteoglycans are Essential for FGF Receptor Signaling During *Drosophila* Embryonic Development

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Running title: Role of HSPGs in FGF signaling

Key Words: *Drosophila*, FGF, heparan sulfate glycosaminoglycan, mesoderm, trachea, cell migration

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Summary

The *Drosophila sugarless* and *sulfateless* genes encode enzymes required for the biosynthesis of heparan sulfate glycosaminoglycans. Biochemical studies have shown that heparan sulfate glycosaminoglycans are involved in signaling by fibroblast growth factor receptors, but evidence for such a requirement in an intact organism has not been available. We now demonstrate that *sugarless* and *sulfateless* mutant embryos have phenotypes similar to those lacking the functions of two *Drosophila* fibroblast growth factor receptors, Heartless and Breathless. Moreover, both Heartless- and Breathless-dependent MAPK activation is significantly reduced in embryos which fail to synthesize heparan sulfate glycosaminoglycans. Consistent with an involvement of Sulfateless and Sugarless in fibroblast growth factor receptor signaling, a constitutively activated form of Heartless partially rescues *sugarless* and *sulfateless* mutants, and dosage-sensitive interactions occur between *heartless* and the heparan sulfate glycosaminoglycan biosynthetic enzyme genes. We also find that overexpression of Branchless, the Breathless ligand, can partially overcome the requirement of Sugarless and Sulfateless for Breathless activity. These results provide the first genetic evidence that heparan sulfate glycosaminoglycans are essential for fibroblast growth factor receptor signaling in a well defined developmental context, and support a model in which heparan sulfate glycosaminoglycans facilitate fibroblast growth factor ligand and/or ligand-receptor oligomerization.

Introduction

The fibroblast growth factors (FGFs) comprise a large family of proteins that act as key intercellular signals in a wide range of developmental and pathological processes (Hanahan and Folkman, 1996; Martin, 1998; Wilkie et al., 1995; Yamaguchi and Rossant, 1995). The biological functions of FGFs include the regulation of cell proliferation, differentiation, survival, motility and tissue patterning. Both biochemical and genetic studies demonstrate that FGFs relay their signals through high affinity transmembrane protein tyrosine kinase receptors (Mason, 1994; Wilkie et al., 1995). By binding to the extracellular domains of these receptor tyrosine kinases (RTKs), FGFs induce the formation of receptor homo- or heterodimers. FGF receptor (FGFR) oligomerization results in RTK transphosphorylation followed by the activation of a Ras-dependent intracellular signal transduction pathway (Fantl et al., 1993).

In addition to its high affinity receptor, biochemical studies indicate that heparin/heparan sulfate proteoglycans (HSPGs) act as low affinity FGF co-receptors that facilitate FGF signal transduction (Mason, 1994; Ornitz et al., 1992; Rapraeger et al., 1991; Schlessinger et al., 1995; Spivak-Kroizman et al., 1994; Yayon et al., 1991). HSPGs are ubiquitous macromolecules that are associated with the cell surface and with the extracellular matrix (Bernfield et al., 1992; David, 1993; Kjellén and Lindahl, 1991; Yanagishita and Hascall, 1992). HSPGs consist of a protein core to which heparin/heparan sulfate glycosaminoglycan (HS GAG) is attached. The function of HSPGs in FGF signaling is mediated by HS GAG chains which are usually highly sulfated and negatively charged. While the precise mechanism by which HS GAGs participate in FGFR activation remains unclear, a variety of biochemical studies suggest that HS GAGs may stabilize or induce the formation of FGF dimers or a

ternary complex composed of ligand plus high and low affinity receptors (DiGabriele et al., 1998; Faham et al., 1996; Herr et al., 1997; Ornitz et al., 1992, 1995; Spivak-Kroizman et al., 1994; Venkataraman et al., 1996; Zhu et al., 1993). Although there is strong *in vitro* evidence implicating HS GAGs in FGFR signaling, there is as yet no *in vivo* genetic support for this hypothesis.

Two FGFRs, Heartless (Htl) and Breathless (Btl), have been characterized in *Drosophila*. Genetic analyses have established that each of these receptors has distinct expression patterns and developmental functions during embryogenesis. Htl is expressed uniformly in the early embryonic mesoderm where it is required for the normal dorsolateral migration of mesodermal cells following gastrulation (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998b; Shishido et al., 1993; Shishido et al., 1997). Htl expression is modulated after mesoderm migration is complete, and its continued activity is essential for the specification of particular cardiac and muscle cell fates (Carmena et al., 1998; Michelson et al., 1998b). Btl is expressed in the tracheal system as well as in a subset of cells in the CNS midline (Klämbt et al., 1992; Shishido et al., 1993). Both of these cell types depend on Btl for their specific patterns of migration. For example, in *btl* mutant embryos, the primary specification of tracheal cells is normal but these cells fail to migrate properly, leading to profound defects in the formation of the tracheal tree (Klämbt et al., 1992). Btl also is required for the determination of specialized cells at the ends of primary tracheal branches that initiate the formation of higher order branching (Lee et al., 1996; Reichman-Fried and Shilo, 1995). While the ligand for Htl has not yet been identified, the Btl ligand is encoded by *branchless* (*bnl*; Sutherland et al., 1996). Mutations in *bnl* are associated with defects in tracheal morphogenesis that are virtually identical to those seen in *btl* mutants. Bnl is expressed dynamically in clusters of cells that are positioned so as to guide the outgrowth and subsequent

branching of neighboring tracheal cells. Ectopic expression of Bnl can redirect tracheal cell outgrowth and branch formation, substantiating the hypothesis that spatially localized activation of the Btl receptor is necessary for normal tracheal morphogenesis (Lee et al., 1996; Sutherland et al., 1996). The characterization of FGFRs and their ligands by a combination of genetic as well as molecular approaches in *Drosophila* provides a very useful paradigm for studying the biological functions of FGFs and for identifying other genetic components involved in FGF signaling.

We have recently identified and characterized two *Drosophila* mutations, *sugarless* (*sgl*, also known as *kiwi* and *suppenkasper*; Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997) and *sulfateless* (*sfl*; Lin and Perrimon, 1999) which encode the homologs of UDP-D-glucose dehydrogenase and heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively. These enzymes are critical for the biosynthesis and modification of HS GAGs, and the corresponding mutants provide an *in vivo* model for examining the involvement of these molecules in FGFR signaling. In particular, we have used these mutants to test the hypothesis that HSPGs act as FGF co-receptors by determining whether *sfl* or *sgl* null embryos exhibit phenotypes characteristic of the high affinity FGFR mutants, *htl* and *btl*, whether *sfl* and *sgl* interact genetically with *htl*, and whether Htl- and Btl-dependent signaling pathways are activated in *sfl* and *sgl* null embryos. In this report, we demonstrate that the complete loss of both the maternal and zygotic components of either *sfl* or *sgl* expression is associated with profound defects in mesoderm and tracheal cell migration. Moreover, both the Htl- and Btl-dependent activation of MAPK that is seen normally in migrating mesodermal and tracheal cells is undetectable in *sfl* and *sgl* mutant embryos. These findings provide the first genetic evidence that HS GAGs are essential for signaling by FGFRs during embryonic development.

Materials and Methods

Drosophila strains

The following *Drosophila* strains were employed: htl^{AB42}, htl^{YY262} (Gisselbrecht et al., 1996; Michelson et al., 1998a), $sfl^{l(3)03844}$ (Lin and Perrimon, 1999), $sgl^{l(3)08310}$ (Häcker et al., 1997), btl^{LG19} (Klämbt et al., 1992), bnl^{P1} (Sutherland et al., 1996), wg^{CX4} (Bejsovec and Wieschaus, 1993; van den Heuvel et al., 1993), *69B*-Gal4 (Brand and Perrimon 1993), *twi*-Gal4 (Greig and Akam, 1993), UAS-Bnl (Sutherland et al., 1996), UAS-Htl^{Act} (Michelson et al., 1998a) and *trh-LacZ* (Wilk et al., 1996). htl^{AB42},$sfl^{l(3)03844}$, $sgl^{l(3)08310}$, btl^{LG19} , bnl^{P1} and wg^{CX4} are all null alleles by genetic and/or molecular criteria. *Oregon R* was used as a wild type reference strain.

Generation of females with *sfl* and *sgl* germline clones

Females with germline clones were generated using the autosomal "FLP-DFS" technique (Chou and Perrimon, 1996). Briefly, virgin females of the genotype *sfl* (or *sgl*) FRT^{2A}/*TM3, Sb* were mated with males of the genotype *y w* FLP^{22}/+, FRT^{2A} $P[ovo^{D1}]$/*TM3, Sb*. The resulting progeny were heat shocked at 37°C for 2 hrs during the larval stages of development, and *y w* FLP^{22}/+; *sfl* (or *sgl*) FRT^{2A}/FRT^{2A} $P[ovo^{D1}]$ females carrying *sfl* (or *sgl*) homozygous germline clones were selected. Such females were mated to *sfl* (or *sgl*)/*TM3-ftzLacZ* males, and maternal/zygotic null embryos were identified by the absence of *LacZ* expression.

Antibody staining

Fixation of embryos, antibody staining and embryo sectioning were performed as described (Gisselbrecht et al., 1996; Michelson et al., 1998a; Patel, 1994). Anti-tracheal lumen antibody MAb2A12 was obtained from the Developmental Studies Hybridoma Bank, anti-β-galactosidase antibody from Cappel and Promega, and anti-diphospho-MAPK antibody from Sigma.

Ectopic expression experiments

Targeted ectopic expression was accomplished using the Gal4/UAS system (Brand and Perrimon 1993). Chromosomes bearing Gal4 and UAS insertions were combined with appropriate mutations using standard genetic crosses. Mesodermal and ectodermal expression were achieved with *twi*-Gal4 and *69B*-Gal4 lines, respectively, in both wild type and mutant genetic backgrounds.

Quantitation of mesodermal phenotypic severity

The hypomorphic htl^{YY262} mutation was recombined with null alleles of *sfl* and *sgl*. Embryos from stocks containing these recombinant chromosomes maintained over a *LacZ*-marked balancer were collected and double stained with antibodies against both Eve and β-galactosidase. Embryos homozygous for the zygotic loss of both genes were identified as lacking *LacZ* expression. Identical experiments were undertaken with each of the single zygotic mutants, and Eve expression in dorsal mesodermal cells was quantitated for each genotype, as previously described (Michelson et al., 1998a,b). The statistical significance of the

difference in Eve expression for each pairwise comparison was calculated using both one-tailed *z*- and *t*-tests.

Results

The genetics of HSPG biosynthesis in *Drosophila*

In a screen to characterize the maternal effects of zygotic lethal mutations (Perrimon et al., 1996), two mutants, *sfl* and *sgl*, were isolated on the basis of their abnormal embryonic segmentation phenotypes. Embryos lacking maternal germline-derived *sfl* or *sgl* activity, as well as the paternally derived zygotic activity of these genes (referred to hereafter as *sfl* or *sgl* null embryos), exhibit a cuticle phenotype similar to that of the *wingless* (*wg*) segment polarity mutant (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). *sgl* encodes a homolog of bovine UDP-glucose dehydrogenase (Hempel et al., 1994) which catalyzes the conversion of UDP-D-glucose to UDP-D-glucuronic acid, an essential substrate for GAG biosynthesis. Consistent with a critical role for Sgl in the synthesis of proteoglycans in *Drosophila*, prior biochemical experiments have established that Syndecan and Dally/Glypican lack GAG chains in *sgl* null embryos (Haerry et al., 1997) or homozygous *sgl* zygotic mutant third instar larvae (Tsuda et al., 1999), respectively. Moreover, injection of either UDP-glucuronic acid or heparan sulfate into *sgl* null embryos rescues the *wg*-like segment polarity defect, and injection of heparinases I and III (but not chondroitinase ABC) into wild type embryos phenocopies loss of *sgl* function (Binari et al., 1997). These findings provide strong evidence that Sgl is essential for the biosynthesis of HS GAG chains which, in turn, are required for Wg signaling.

Drosophila Sfl (Lin and Perrimon, 1999) has 51% and 53% amino acid identity to rat and mouse heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively (Eriksson et al., 1994; Hashimoto et al., 1992; Orellana et al., 1994). This enzyme catalyzes the N-deacetylation and N-sulfation of polymerized heparan (as opposed to chondroitin or deramatan) GAGs, the key step that initiates further GAG modifications (for review see Kjellén and Lindahl, 1991). Since the sulfation and epimerization of HS GAGs provide structural identity as well as the negative charge that is critical for their interaction with proteins, loss of Sfl activity will result in the generation of unmodified HS GAGs, thereby impairing normal HSPG functions. Indeed, similar to the case in *sgl* mutants (Tsuda et al., 1999), the HS GAG-modified Dally protein is markedly reduced in *sfl* zygotic mutant larvae, indicating that, in the absence of Sfl activity, HS GAG biosynthesis is abnormal (Lin and Perrimon, 1999). Together with the strong *wg*-like segment polarity phenotype associated with loss of *sfl* function, these results provide compelling evidence that Sfl is essential for the production of proteoglycans involved in Wg signaling (Lin and Perrimon, 1999). Moreover, the specificity of Sfl for heparan-containing GAGs distinguishes the type of GAG chain that participates in this signaling pathway. In summary, phenotypic analyses of *sfl* and *sgl* mutants can be used to study the roles of HSPGs in normal developmental processes.

sfl and _sgl_ mutants phenocopy the mesoderm migration defect associated with loss of _htl_ function

After invaginating through the ventral furrow at gastrulation, Twist- (Twi) expressing mesodermal cells migrate along the ectoderm in a dorsolateral direction. By late stage 9, the mesoderm is composed of an inner monolayer of cells that extends from the ventral midline to the dorsal edge of the ectoderm (Bate, 1993;

Leptin, 1995); Fig. 1A,E). In *htl* mutant embryos, gastrulation is normal but mesoderm migration fails to occur properly, resulting in an irregular dorsal margin of Twi-positive cells and a relative accumulation of these cells in ventral and lateral positions (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Michelson et al., 1998b; Fig. 1B,F). A similar phenotype occurs in *sfl* and *sgl* null embryos (Fig. 1C,D,G,H). Of note, mesoderm migration is normal in *wg* mutant embryos (data not shown), suggesting that this effect of *sfl* and *sgl* is not due to an influence on Wg signaling, as is the case for the segmentation effects of these genes (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999).

The finding that mesoderm migration depends on *sfl* and *sgl*, as well as on *htl*, raises the possibility that HS GAG biosynthesis is required for signaling by the Htl FGFR. If, as has been suggested, HS GAGs participate in the activation of FGFRs (Ornitz et al., 1992; Rapraeger et al., 1991; Schlessinger et al., 1995; Yayon et al., 1991), then a constitutively active form of Htl should bypass the requirement of *sfl* and *sgl* for mesoderm migration. We tested this hypothesis by targeting the expression of activated Htl to the mesoderm of *sfl* or *sgl* null embryos using the Gal4/UAS system (Brand and Perrimon, 1993). We previously reported the construction of such an activated receptor in which the extracellular domain of wild type Htl is replaced by the dimerization domain of the bacteriophage λ cI repressor (Michelson et al., 1998a). As with other RTKs, this manipulation generates constitutive, ligand-independent receptor activity (Lee et al., 1996; Michelson et al., 1998a; Queenan et al., 1997). In otherwise wild type embryos, *twi*-Gal4-mediated ectopic expression of activated Htl has no significant effect on the migration of mesodermal cells (Michelson et al., 1998a; Fig. 1I). However, activated Htl is able to weakly restore mesoderm migration in a null *htl* mutant (Michelson et al., 1998a; Fig. 1J). Similarly, activated Htl partially rescues the migration defect of *sfl* and *sgl* null embryos (Fig. 1K,L). Only

partial rescue is seen in all mutant backgrounds due to the relatively weak constitutive activation of Htl that is achieved by spontaneous dimerization (Michelson et al., 1998a). In addition, constitutive Htl does not reproduce the graded activity of this receptor that occurs during normal mesoderm migration (Gabay et al., 1997b; Michelson et al., 1998a). The timing of constitutive Htl expression induced by *twi*-Gal4 is unlikely to be a contributing factor since the equivalent expression of wild type Htl completely rescues a null *htl* allele (Michelson et al., 1998b). In summary, these genetic epistasis experiments suggest that Htl acts downstream of Sfl and Sgl in migrating mesodermal cells.

If HS GAGs participate in the activation of Htl, then *sfl* and *sgl* should exhibit dosage-sensitive genetic interactions with *htl*. We investigated this possibility using a quantitative assay that is based on the expression of *even skipped* (*eve*) in dorsal mesodermal progenitor cells (Buff et al., 1998; Carmena et al., 1998; Frasch et al., 1987; Michelson et al., 1998a,b). The development of these Eve-expressing cardiac and somatic muscle cells depends on Htl for both mesoderm migration and cell fate specification. In wild type embryos, Eve is found in segmentally repeated groups of cells that are confined to the dorsal mesoderm (Fig. 2A). All such cells are missing in a null *htl* mutant (Gisselbrecht et al., 1996). However, in a *htl* hypomorph, mesoderm migration and subsequent cell fate specification are only partially disrupted, permitting some dorsal Eve expression to occur (Fig. 2B; Michelson et al., 1998b). In embryos in which only the zygotic activity of *sfl* or *sgl* is absent, Eve expression is entirely normal, reflecting the strong maternal contribution of these genes (Fig. 2C and data not shown). However, when complete zygotic loss of *sfl* or *sgl* is combined with homozygosity for the *htl* hypomorphic allele, the severity of the Eve phenotype is enhanced (Fig. 2D). Quantitation of the number of Eve-positive hemisegments in *htl*, *sfl*, *sgl*, *htl sfl* and *htl sgl* embryos demonstrated that

the interactions between *htl* and both *sfl* and *sgl* are highly significant (Fig. 2E; $P<10^{-5}$). These results, combined with the above findings that *htl* is epistatic to *sfl* and *sgl*, are consistent with the hypothesis that HS GAGs are essential for Htl activation during mesoderm migration in the *Drosophila* embryo.

sfl and _sgl_ are required for Btl-dependent tracheal cell migration

Given the genetic evidence that Sfl and Sgl are required for Htl FGFR signaling in the mesoderm, we next determined whether HS GAG biosynthesis is also involved in signaling by Bnl and Btl during trachea development. Since Wg function is compromised in *sgl* and *sfl* null mutant embryos, and *wg* loss-of-function itself leads to abnormal tracheal morphogenesis secondary to an accompanying segmentation defect (see below), it is difficult to solely correlate the tracheal cell migration phenotype of *sgl* and *sfl* null mutants with Bnl/Btl signaling. We therefore analyzed tracheal cell migration in embryos deficient only in the zygotic functions of *sfl* and *sgl*. Segmentation and the expression of Engrailed are normal in such embryos, indicating that Wg signaling is unaffected (Perrimon et al., 1996; Häcker et al., 1997; Lin and Perrimon, 1999).

The tracheal system of the *Drosophila* embryo forms by a sequential series of branching steps that can be visualized by following the expression of an enhancer trap in the *trachealess* (*trh*) gene (Isaac and Andrew, 1996; Wilk et al., 1996); Fig. 3A). In stage 13 embryos homozygous for loss of the zygotic functions of either *sfl* or *sgl*, the early steps in tracheal branching are significantly perturbed (Fig. 3B,C). By late stage 15, wild type embryos have developed an extensive tracheal network in which the dorsal and lateral trunk branches have fused and additional primary and

secondary branches have formed (Samakovlis et al., 1996); Fig. 3D). In either *sfl* or *sgl* zygotic mutants, tracheal branch formation is incomplete, as revealed by the presence of large gaps in the dorsal and lateral trunks, as well as stalled ganglionic branches (Figure 3E,F). The penetrance of this phenotype is incomplete and the expressivity is variable in both *sfl* and *sgl* zygotic mutants; 16% of *sfl* (n = 245) and 7% of *sgl* (n = 198) zygotic mutant embryos exhibit some degree of abnormal tracheal morphogenesis, ranging from one to all segments having breaks in the dorsal trunk. In contrast, in *btl* and *bnl* null mutant embryos, tracheal cell migration is more severely affected and virtually no branches form from the initial tracheal invaginations (Klämbt et al., 1992; Sutherland et al., 1996; Fig. 3G). The partial disruption of the tracheal tree seen with zygotic loss of *sfl* and *sgl* is reminiscent of the defects observed in hypomorphic mutants of *btl, bnl* and *heartbroken (hbr)/downstream of FGF receptor (dof)/ stumps*, a gene that encodes a specific effector of FGF receptor signaling (Klämbt et al., 1992; Michelson et al., 1998a; Sutherland et al., 1996; Vincent et al., 1998; Imam et al., 1999). The relatively weak zygotic *sfl* and *sgl* tracheal phenotypes are most likely due to partial rescue by the maternal expression of these genes, as previously noted for their mesodermal activities. The more severe tracheal defects that occur in *sfl* and *sgl* null embryos are consistent with this suggestion (see below). In summary, the present findings implicate Sfl- and Sgl-dependent HS GAG biosynthesis in signaling by the Btl FGFR.

Htl- and Btl-dependent MAPK activation depends on *sfl* and *sgl*

Htl and Btl are RTKs that transduce their intracellular signals by the conserved Ras/MAPK cascade (Cobb and Goldsmith, 1995; Seger and Krebs, 1995). As a result, RTK activity can be visualized in developing tissues with an antibody specific for the diphosphorylated, activated form of MAPK (Gabay et al., 1997a,b).

Using this approach, diphospho-MAPK is observed at the leading edge of the migrating mesoderm in wild type embryos (Gabay et al., 1997b; Michelson et al., 1998a; Vincent et al., 1998; Fig. 4A). In either *htl* or *hbr/dof/stumps* mutants, this expression of diphospho-MAPK is undetectable (Michelson et al., 1998a; Vincent et al., 1998; Fig. 4B). Similarly, the Htl-dependent mesodermal localization of diphospho-MAPK is below the level of detection in both *sfl* and *sgl* null embryos (Fig. 4C,D). In contrast, epidermal growth factor receptor (EGFR)-dependent MAPK activation in the ventral epidermis and amnioserosa does not require either *sfl* or *sgl*, consistent with the specific involvement of these genes in FGFR signaling.

During stage 11, MAPK is activated by Btl in the tracheal pits (Gabay et al., 1997b; Fig. 4E). As expected, this expression of diphospho-MAPK is markedly reduced in *btl* and *bnl* mutant embryos (Fig. 4F,G). The FGFR-specific signal transducer Hbr/Dof/Stumps also is required for MAPK activation in the tracheal pits (Michelson et al., 1998a; Vincent et al., 1998). In contrast, in *wg* mutants MAPK activation by Btl is unaffected, although the normal spacing between the tracheal pits is reduced due to the associated segmentation defect (Fig. 4H). As with *btl*, *bnl* and *hbr/dof*/stumps, Btl-dependent MAPK activation is significantly decreased in *sfl* or *sgl* null embryos (Fig. 4I,J), whereas the earlier EGFR-dependent expression of diphospho-MAPK in the tracheal placodes is unaffected in each of these mutants (Michelson et al., 1998a; data not shown). These *in situ* patterns of MAPK activation provide direct evidence that Sfl and Sgl are required for signaling by the two *Drosophila* FGFRs, Htl and Btl, independent of the requirement for HS GAGs in Wg function.

Overexpression of Bnl partially overcomes the requirement of *sfl* and *sgl* for tracheal cell migration

It has been suggested that monomeric FGF molecules are capable of self-assembling into dimers and higher order oligomers but, at physiological concentrations, require a HS GAG to stabilize this interaction (Sasisekharan et al., 1997; Venkataraman et al., 1996). If this is the case, then elevated levels of the growth factor may at least partially overcome the need for the HS GAG to generate a biological response. We tested this possibility by assessing the effects on tracheal cell migration of overexpressing Bnl, the Btl ligand, in *sfl* and *sgl* null embryos.

In an otherwise wild type genetic background, high level ectopic ectodermal expression of Bnl hyperactivates Btl, leading to an inhibition of primary tracheal branching, as well as the overproduction of secondary and terminal branches (Sutherland et al., 1996; Michelson et al., 1998a; Fig. 5A,B). These effects of ectopic Bnl are weakly suppressed in the absence of zygotic *sfl* and *sgl* functions (Fig. 5C,D). In embryos lacking both the zygotic and maternal components of *sfl* and *sgl* expression, virtually no tracheal branches are observed (Fig. 5E,F). This correlates very well with the marked reduction in diphospho-MAPK expression that is seen at earlier stages in *sfl* and *sgl* null embryos (Fig. 4I,J). However, in *sfl* and *sgl* null embryos in which Bnl is ectopically expressed, there is a partial recovery of tracheal branching (Fig. 5G,H). Although the tracheal phenotype of *sfl* and *sgl* null embryos reflects the combined loss of Wg and Btl signaling, it is noteworthy that tracheal morphogenesis is not completely inhibited in *wg* as it is in null *sfl* and *sgl* mutants (compare Fig. 5E,F and I). Moreover, ectopic Bnl in the absence of *wg* function leads to a very marked increase in fine tracheal branching, very similar to the effect of ectopic Bnl in wild type embryos (compare Fig. 5B and J). Thus, the severe tracheal

phenotype associated with complete loss of *sfl* or *sgl* function primarily is attributable to an involvement of HS GAGs in Btl rather than in Wg signaling. The ability of Bnl overexpression to partially bypass the requirement for Sfl and Sgl is therefore consistent with a role for HS GAGs in stabilizing or facilitating FGF self-association (Zhu et al., 1993; Ornitz et al., 1995; Faham et al., 1996; Venkataraman et al., 1996; Sasisekharan et al., 1997; DiGabriele et al., 1998). Such a HS GAG-FGF dimer complex would, in turn, facilitate the dimerization of high affinity FGFRs, a prerequisite for receptor activation and the transmission of intracellular signals.

Discussion

We have shown that two enzymes involved in the biosynthesis of HS GAGs are essential for signaling by both of the known *Drosophila* FGFRs, Htl and Btl. Loss of either *sfl* or *sgl* function leads to defects in the migration of mesodermal and tracheal cells during embryogenesis. In addition, Htl- and Btl-dependent activation of MAPK is markedly reduced in *sfl* and *sgl* null embryos. Taken together, these findings provide the first genetic evidence that HSPGs play a central role in FGFR signaling in a well-defined developmental context. A similar genetic approach has been used to establish that HSPGs are critical components of Wg signaling in *Drosophila* (Binari et al., 1997; Häcker et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999).

Several mechanisms have been proposed for how HSPGs participate in FGFR signaling. In one model, the binding of FGF to abundant but low affinity HS GAGs on the cell surface limits the free diffusion of the ligand, thereby increasing its local concentration and the probability that it will interact with less abundant, high affinity signaling receptors (Schlessinger et al., 1995). Other studies have identified

distinct HS GAG binding sites on both FGF (Blaber et al., 1996; Eriksson et al., 1991; Zhang et al., 1991) and high affinity FGFRs (Kan et al., 1993; Pantoliano et al., 1994), suggesting that the latter two components form a ternary complex with a proteoglycan. Direct interaction between the proteoglycan and FGFR, in addition to growth factor binding, may therefore be required for maximal receptor activation. HS GAGs also may promote the formation of FGF dimers or higher order oligomers, thereby facilitating FGFR dimerization and activation (Zhu et al., 1993; Spivak-Kroizman et al., 1994; Ornitz et al., 1995; Faham et al., 1996; Digabriele et al., 1998).

A variation of the last model proposes that FGF monomers are capable of self-associating, a process that is stabilized by HS GAGs (Venkataraman et al., 1996; Herr et al., 1997; Sasisekharan et al., 1997). This hypothesis predicts that elevated levels of FGF should compensate at least in part for a loss of dimer stabilization mediated by HS GAGs. The ability of Bnl overexpression to induce some tracheal branching in the complete absence of Sfl and Sgl activities is consistent with this last possibility, although it does not rule out an additional involvement of HS GAGs or the core protein of a proteoglycan in some other aspect of Btl activation. In the case of human FGF-2, self-association in the absence of HS GAGs has been observed by mass spectrometry and biochemical assays at physiological concentrations of ligand. In addition, elevated FGF-2 levels exert biological effects on tissue culture cells that fail to synthesize HS GAGs (J. Davis, G. Venkataraman, Z. Shriver, A. Periathamby and R. Sasisekharan, manuscript submitted). Our Bnl overexpression experiments are in agreement with the latter data for FGF-2. Together, these findings support the proposal that HS GAGs function to stabilize the FGF dimers or higher order oligomers that are formed by a self-association mechanism (Venkataraman et al., 1996; Sasisekharan et al., 1997). Interestingly, ectopic expression of ligand also is able

to overcome the requirement of HS GAGs in Wg signaling, although in this case the HS GAGs may be more important for increasing the local concentration of growth factor at the cell surface than for facilitating ligand dimerization (Häcker et al., 1997; Lin and Perrimon, 1999).

Under normal conditions, the concentrations of FGFs may be limiting, necessitating the presence of HS GAGs to augment or stabilize ligand dimerization and subsequent FGFR activation. This is of particular significance for Btl signaling since *bnl*, which encodes its ligand, is known to be haploinsufficient (Sutherland et al., 1996), and overexpression of Bnl partially bypasses the requirement for *sfl* and *sgl* in the promotion of tracheal branching. Thus, HS GAGs may insure that a requisite FGFR activation threshold is surpassed when the amount of available ligand is normally low. Such a mechanism could additionally expand the sensitivity or spectrum of responses that can be achieved by small localized differences in growth factor concentrations. This is particularly relevant to Bnl which is expressed in a highly dynamic pattern during normal tracheal morphogenesis, a pattern that, when perturbed, leads to severe defects in tracheal outgrowth (Sutherland et al., 1996). Local differences in Btl activity also dictate the sites at which secondary branches normally form, a process to which HSPG regulation might contribute by generating a zone of cells that are highly responsive to Bnl in the vicinity of the ligand signaling center (Hacohen et al., 1998). Similarly, HSPG-mediated ligand dimerization could play a role in the graded activation of Htl that occurs during embryonic mesoderm migration (Gabay et al., 1997b). Modulation of RTK signaling strength also has been implicated in the generation of mesodermal progenitor identities (Buff et al., 1998).

There are two well characterized HSPGs in *Drosophila*, Dally, a Glypican-like cell surface molecule that has been implicated in both Decapentaplegic and Wg signaling (Nakato et al., 1995; Lin and Perrimon, 1999; Tsuda et al., 1999), and a transmembrane proteoglycan related to the vertebrate Syndecan family (Spring et al., 1994). It has been suggested that syndecans particpate in signaling by vertebrate FGFRs (Bernfield et al., 1992; Dealy et al., 1997; Steinfeld et al., 1996), although other HSPGs may also be involved in this process (Aviezer et al., 1994; Aviezer et al., 1997; Sherman et al., 1998; Steinfeld et al., 1996). It is also possible that different HSPGs could be specific for particular FGF ligand-receptor combinations in individual tissues or at distinct developmental stages. Genetic analysis in *Drosophila* should provide a useful approach for addressing these important questions.

The present findings provide new insight into the mechanisms that regulate FGFR signaling *in vivo*. A more complete understanding of the function of HSPGs in Htl and Btl activation must await definitive identification of the specific proteoglycans involved, as well as structural and biochemical studies of the complexes formed between the high and low affinity receptors together with the corresponding ligands.

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References

Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G. and Yayon, A. (1994). Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. *Cell* **79**, 1005-1013.

Aviezer, D., Renato, I. V., Noonan, D. M. and Yayon, A. (1997). Suppresion of autocrine and paracrine functions of basic fibroblast growth factor by stable expression of perlecan antisense cDNA. *Mol.Cell.Biol.* **17**, 1938-1946.

Bate, M. (1993). The mesoderm and its derivatives. In *The development of Drosophila melanogaster,* (ed. M. Bate and A. Martinez Arias), pp. 1013-1090: Cold Spring Harbor Laboratory Press.

Beiman, M., Shilo, B. Z. and Volk, T. (1996). Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Dev.* **10**, 2993-3002.

Bejsovec, A. and Wieschaus, E. (1993). Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. *Development* **119**, 501-517.

Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. and Lose, E. J. (1992). Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* **8**, 365-393.

Binari, R. C., Staveley, B. E., Johnson, W. A., Godavarti, R., Sasisekharan, R. and Manoukian, A. S. (1997). Genetic evidence that heparin-like glycosaminoglycans are involved in *wingless* signaling. *Development* **124**, 2623-2632.

Blaber, M., DiSalvo, J. and Thomas, K. A. (1996). X-ray crystal structure of human acidic fibroblast growth factor. *Biochemistry* **35**, 2086-2094.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Buff, E., Carmena, A., Gisselbrecht, S., Jiménez, F. and Michelson, A. M. (1998). Signalling by the *Drosophila* epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development* **125**, 2075-2086.

Carmena, A., Gisselbrecht, S., Harrison, J., Jiménez, F. and Michelson, A. M. (1998). Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Dev.* **12**, 3910-3922.

Chou, T.-b. and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. *Genetics* **144**, 1673-1679.

Cobb, M. H. and Goldsmith, E. J. (1995). How MAP kinases are regulated. *J. Biol. Chem.* **270**, 14843-14846.

David, G. (1993). Integral membrane heparan sulfate proteoglycans. *FASEB J.* **7**, 1023-1030.

Dealy, C. N., Seghatoleslami, M. R., Ferrari, D. and Kosher, R. A. (1997). FGF-stimulated outgrowth and proliferation of limb mesoderm is dependent on syndecan-3. *Dev. Biol.* **184**, 343-350.

DiGabriele, A. D., Lax, I., Chen, D. I., Svahn, C. M., Jaye, M., Schlessinger, J. and Hendrickson, W. A. (1998). Structure of a heparin-linked biologically active dimer of fibroblast growth factor. *Nature* **393**, 812-817.

Eriksson, A. E., Cousens, L. S., Weaver, L. H. and Matthews, B. W. (1991). Three-dimensional structure of human basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **88**, 3441-3445.

Eriksson, I., Sandback, D., Lindahl, U. and Kjellén, L. (1994). cDNA cloning and sequencing of mouse mastocytoma glucosaminyl *N*-deacetylase/*N*-sulfotransferase, an enzyme involved in the biosynthesis of heparin. *J. Biol. Chem.* **269**, 10438-10443.

Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J. and Rees, D. C. (1996). Heparin structure and interactions with basic fibroblast growth factor. *Science* **271**, 1116-1120.

Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993). Signalling by receptor tryrosine kinases. *Annu. Rev. Biochem.* **62**, 453-481.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the *even-skipped* protein of *Drosophila*. *EMBO J.* **6**, 749-759.

Gabay, L., Seger, R. and Shilo, B.-Z. (1997a). In situ activation pattern of *Drosophila* EGF receptor pathway during development. *Science* **277**, 1103-1106.

Gabay, L., Seger, R. and Shilo, B.-Z. (1997b). MAP kinase in situ activation atlas during *Drosophila* embryogenesis. *Development* **124**, 3535-3541.

Gisselbrecht, S., Skeath, J. B., Doe, C. Q. and Michelson, A. M. (1996). Heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Dev.* **10**, 3003-3017.

Greig, S. and Akam, M. (1993). Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. *Nature* **362**, 630-632.

Häcker, U., Lin, X. and Perrimon, N. (1997). The *Drosophila sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565-3523.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. and Krasnow, M. A. (1998). *sprouty* encodes a novel antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways. *Cell* **92**, 253-263.

Haerry, T. E., Heslip, T. R., Marsh, J. L. and O'Connor, M. B. (1997). Defects in glucuronate biosynthesis disrupt Wingless signaling in *Drosophila*. *Development* **124**, 3055-3064.

Hanahan, D. and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364.

Hashimoto, Y., Orellana, A., Gil, G. and Hirschberg, C. B. (1992). Molecular cloning and expression of rat liver *N*-heparan sulfate sulfotransferase. *J. Biol. Chem.* **267**, 15744-15750.

Hempel, J., Perozich, J., Romavacek, H., Hinich, A., Kuo, I. and Feingold, D. S. (1994). UDP-glucose dehydrogenase from bovine liver: primary structure and relationship to other dehydrogenases. *Protein Sci.* **3**, 1074-1080.

Herr, A. B., Ornitz, D. M., Sasisekharan, R., Venkataraman, G. and Waksman, G. (1997). Heparin-induced self-association of fibroblast growth factor-2. *J. Biol. Chem.* **272**, 16382-16389.

Imam, F., Sutherland, D., Huang, W. and Krasnow, M. A. (1999) *stumps*, a *Drosophila* gene required for fibroblast growth (FGF)-directed migrations of tracheal and mesodermal cells. *Genetics* **152**, 307-318.

Isaac, D. D. and Andrew, D. J. (1996). Tubulogenesis in *Drosophila*: a requirement for the *trachealess* gene product. *Genes Dev.* **10**, 103-117.

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J. and McKeehan, W. L. (1993). An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science* **259**, 1918-1921.

Kjellén, L. and Lindahl, U. (1991). Proteoglycans: structures and interactions. *Annu. Rev. Biochem.* **60**, 443-475.

Klämbt, C., Glazer, L. and Shilo, B.-Z. (1992). *breathless*, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.* **6**, 1668-1678.

Lee, T., Hacohen, N., Krasnow, M. and Montell, D. J. (1996). Regulated Breathless receptor tyrosine kinase activity required to pattern cell migration and branching in the *Drosophila* tracheal system. *Genes Dev.* **10**, 2912-2921.

Leptin, M. (1995). *Drosophila* gastrulation: from pattern formation to morphogenesis. *Annu. Rev. Cell Dev. Biol.* **11**, 189-212.

Lin, X. and Perrimon, N. (1999) The heparan sulfate proteoglycan Dally/Glypican regulates Wingless signaling in *Drosophila*. Nature, in press.

Martin, G. (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* **12**, 1571-1586.

Mason, I. J. (1994). The ins and outs of fibroblast growth factors. *Cell* **78**, 547-552.

Michelson, A. M., Gisselbrecht, S., Buff, E. and Skeath, J. B. (1998a). Heartbroken is a specific downstream mediator of FGF receptor signalling in *Drosophila*. *Development* **125**, 4379-4389.

Michelson, A. M., Gisselbrecht, S., Zhou, Y., Baek, K.-H. and Buff, E. M. (1998b). Dual functions of the Heartless fibroblast growth factor receptor in development of the *Drosophila* embryonic mesoderm. *Dev. Genet.* **22**, 212-229.

Nakato, H., Futch, T. A. and Selleck, S. B. (1995). The *division abnormally delayed (dally)* gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. *Development* **121**, 3687-3702.

Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S. J. and Ishihara, M. (1994). Molecular cloning and expression of a glycosaminoglycan *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase from a heparin-producing cell line. *J. Biol. Chem.* **269**, 2270-2276.

Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C.-M. and Waksman, G. (1995). FGF binding and FGF receptor activation by synthetic heparan-derived di- and triaccharides. *Science* **268**, 432-436.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. (1992). Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol.* **12**, 240-247.

Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyke, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D. and Sisk, W. P. (1994). Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry* **33**, 10229-10248.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In *Meths. Cell Biol.*, vol. 44 (ed. L. S. B. Goldstein and E. A. Fyrberg), pp. 445-487. New York: Academic Press.

Perrimon, N., Lanjuin, A., Arnold, C. and Noll, E. (1996). Zygotic lethal mutations with maternal effect phenotypes in *Drosophila melanogaster*. II. Loci on the second and third chromosomes identified by P-element-induced mutations. *Genetics* **144**, 1681-1692.

Queenan, A. M., Ghabrial, A. and Schüpbach, T. (1997). Ectopic activation of *torpedo/Egfr*, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. *Development* **124**, 3871-3880.

Rapraeger, A. C., Krufka, A. and Olwin, B. B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**, 1705-1708.

Reichman-Fried, M. and Shilo, B.-Z. (1995). Breathless, a *Drosophila* FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. *Mechs. Dev.* **52**, 265-273.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D. C., Guillemin, K. and Krasnow, M. A. (1996). Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**, 1395-1407.

Sasisekharan, R., Ernst, S. and Venkataraman, G. (1997). On the regulation of fibroblast growth factor activity by heparin-like glycosaminoglycans. *Angiogenesis* **1**, 45-54.

Schlessinger, J., Lax, I. and Lemmon, M. (1995). Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell* **83**, 357-360.

Seger, R. and Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB J.* **9**, 726-735.

Sherman, L., Wainwright, D., Ponta, H. and Herrlich, P. (1998). A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. *Genes Dev.* **12**, 1058-1071.

Shishido, E., Higashijima, S., Emori, Y. and Saigo, K. (1993). Two FGF-receptor homologues of *Drosophila* - one is expressed in mesodermal primordium in early embryos. *Development* **117**, 751-761.

Shishido, E., Ono, N., Kojima, T. and Saigo, K. (1997). Requirements of DFR1/Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development* **124**, 2119-2128.

Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladburt, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J. and Lax, I. (1994). Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* **79**, 1015-1024.

Spring, J., Paine-Saunders, S. E., Hynes, R. O. and Bernfield, M. (1994). *Drosophila* syndecan: Conservation of a cell-surface heparan sulfate proteoglycan. *Proc. Natl. Acad. Sci. USA* **91**, 3334-3338.

Steinfeld, R., Van Den Berghe, H. and David, G. (1996). Stimulation of fibroblast growth factor receptor-1 occupancy and signaling by cell surface-assocated syndecans and glypican. *J. Cell Biol.* **133**, 405-416.

Sutherland, D., Samakovlis, C. and Krasnow, M. (1996). *branchless* encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091-1101.

Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Leggin, B., Humphrey, M., Olson, S., Futch, T., Siegfried, E., Stam, L. and Selleck, S. B. (1999). A cell surface proteoglycan, Dally, regulates Wingless signaling in *Drosophila. Nature,* in press.

van den Heuvel, M., Harryman-Samos, C., Klingensmith, J., Perrimon, N. and Nusse, R. (1993). Mutations in the segment polarity genes *wingless* and *porcupine* impair secretion or the Wingless protein. *EMBO J.* **12**, 5293-5302.

Venkataraman, G., Sasisekharan, V., Herr, A. B., Ornitz, D. M., Waksman, G., Cooney, C. L., Langer, R. and Sasisekharan, R. (1996). Preferential self-association of basic fibroblast growth factor is stabilized by heparin during receptor dimerization and activation. *Proc. Natl. Acad. Sci. USA 93*, 845-850.

Vincent, S., Wilson, R., Coelho, C., Affolter, M. and Leptin, M. (1998). The *Drosophila* protein Dof is specifically required for FGF signaling. *Mol. Cell* **2**, 515-525.

Wilk, R., Weizman, I. and Shilo, B.-Z. (1996). *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* **10**, 93-102.

Wilkie, A. O. M., Morriss-Kay, G. M., Jones, E. Y. and Heath, J. K. (1995). Functions of fibroblast growth factors and their receptors. *Curr. Biol.* **5**, 500-507.

Yamaguchi, T. P. and Rossant, J. (1995). Fibroblast growth factors in mammalian development. *Curr. Opin. Genet. Dev.* **5**, 485-491.

Yanagishita, M. and Hascall, V. C. (1992). Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **267**, 9451-9454.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841-848.

Zhang, J., Cousens, L. S., Barr, P. J. and Sprang, S. R. (1991). Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1β. *Proc. Natl. Acad. Sci. USA* **88**, 3446-3450.

Zhu, X., Hsu, B. T. and Rees, D. C. (1993). Structural studies of the binding of the anti-ulcer drug sucrose octasulfate to acidic fibroblast growth factor. *Structure* **1**, 27-34.

Figure Legends

Figure 1. *sfl* and *sgl* are required for the dorsolateral migration of the embryonic mesoderm, a requirement that is partially bypassed by constitutive Htl signaling. A-D. Ventral views of late stage 9 embryos of the indicated genotypes immunostained for Twi expression. mat+zyg: an embryo from a germline clone female that also has inherited a mutant paternal chromosome. These embryos therefore lack both the maternal and zygotic functions of *sfl* or *sgl*. E-H. Transverse sections of Twi-stained early stage 10 embryos of the indicated genotypes. Whereas Twi-positive mesodermal cells have reached the dorsal ectoderm in wild type, mesoderm migration fails to occur properly in null *htl* as well as in maternal/zygotic null *sfl* and *sgl* embryos. I-L. *twi*-Gal4-mediated ectopic expression of a constitutively activated form of Htl partially rescues the mesoderm migration defects of null *htl* and maternal/zygotic null *sfl* and *sgl* embryos, but has no effect on migration in an otherwise wild type genetic background (I).

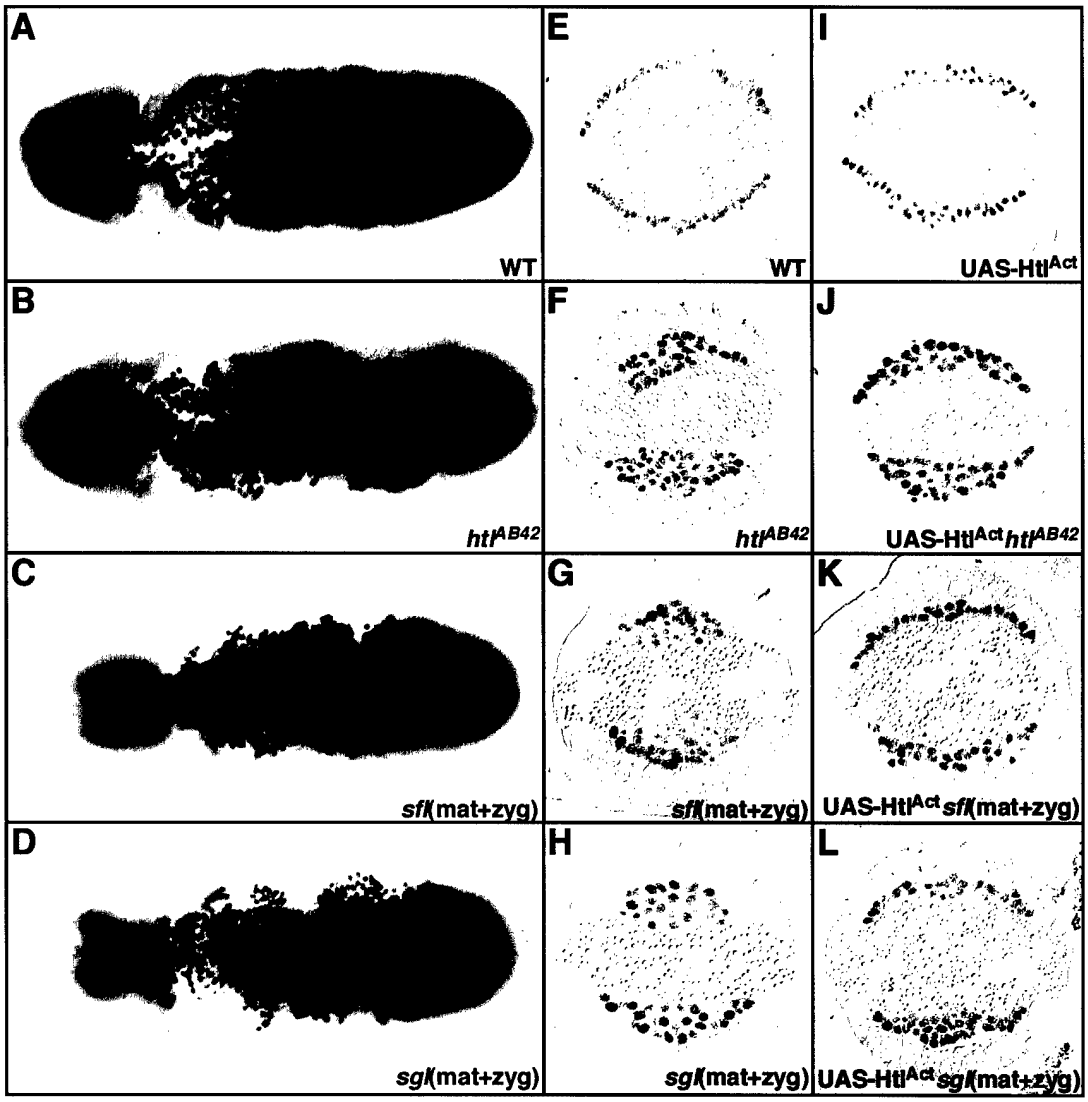
Figure 2. *sfl* and *sgl* interact genetically with *htl*. A-D. Lateral views of stage 11 Eve-stained embryos of the indicated genotypes. htl^{YY262}: a *htl* hypomorphic allele. zyg: an embryo which is homozygous for the zygotic loss of *sfl* or *sgl* function but in which maternal expression of the corresponding gene is normal. E. Quantitation of Eve expression in each genetic background. The numbers above the bars represent the average number of hemisegments per embryo of the indicated genotypes in which Eve-positive cells develop. Each error bar indicates the standard error of the mean for that genotype. Eve expression was scored in the second and third thoracic and first seven abdominal segments on both sides of each embryo. At least 80 embryos of each genotype were scored. The Eve pattern is unaltered by zygotic loss of either *sfl* or *sgl* due to the normal maternal expression of these genes. In a *htl*

hypomorph, only a partial reduction in Eve expression occurs, a phenotype that is significantly ($P<10^{-5}$) enhanced by the zygotic loss of either *sfl* or *sgl*.

Figure 3. *sfl* and *sgl* are required for tracheal cell migration. A-C. Lateral views of stage 13 embryos of the indicated genotypes that also bear an enhancer trap P element insertion in the *trh* gene stained with an antibody against β-galactosidase. D-G. Lateral views of late stage 15 embryos stained for expression of an antigen that localizes to the tracheal lumen. Tracheal cell migration is abnormal in embryos deficient in the zygotic functions of *sfl* and *sgl*. This phenotype is incompletely penetrant and its expressivity is variable (see text for details). The embryos shown in panels B,C,E and F represent phenotypes of intermediate severity.

Figure 4. *sfl* and *sgl* are essential for Htl- and Btl-dependent MAPK activation. A-D. Transverse sections of stage 8 embryos immunostained with an antibody specific for the activated or diphosphorylated form of MAPK. In wild type, diphospho-MAPK expression is localized to the leading edge (arrowheads) of the migrating mesoderm (ms). This expression is below the level of detection in null *htl* as well as in maternal/zygotic null *sfl* and *sgl* embyros. However, EGFR-dependent diphospho-MAPK expression in the amnioserosa (as) and ventral epidermis (ve) is not affected in any of these mutants. E-J. Lateral views of stage 11 embryos stained with the diphospho-MAPK-specific antibody. The strong expression that is observed in wild type tracheal pits (tp) is markedly reduced in the corresponding positions in null *btl* and *bnl* as well as in maternal/zygotic null *sfl* and *sgl* embryos. In contrast, diphospho-MAPK is normally expressed in the tracheal pits of *wg* mutant embryos, although these structures are spaced more closely secondary to the *wg* segment polarity phenotype.

Figure 5. Overexpression of Bnl partially rescues tracheal cell migration in *sfl* and *sgl* maternal/zygotic null mutant embryos. Lateral views of late stage 15 embryos immunostained with an antibody against a tracheal lumenal antigen. A. Wild type tracheal pattern. In B,C,D,G,H and J Bnl was ectopically expressed throughout the ectoderm of embryos of the indicated genotypes using the *69B*-Gal4 driver. B. Tracheal branching is markedly perturbed by ectopic Bnl in an otherwise wild type embryo. Primary branching is suppressed and an overabundance of secondary and terminal branches is induced. C,D. In embryos lacking only the zygotic component of *sfl* or *sgl*, the effects of ectopic Bnl are weakly blocked. E,F. Virtually no tracheal branching occurs in maternal/zygotic null *sfl* and *sgl* embryos. G,H. Some tracheal branching is recovered in maternal/zygotic null *sfl* and *sgl* embryos in which Bnl is ectopically expressed at high levels. I. *wg* mutants develop an extensive tracheal network which has an abnormal pattern due to the associated segmentation defect. J. Ectopic Bnl in a *wg* mutant background leads to an overproduction of fine tracheal branches, much as occurs in wild type (compare with B).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G
sfl(mat+zyg)
K
UAS-HtlAct sfl(mat+zyg)
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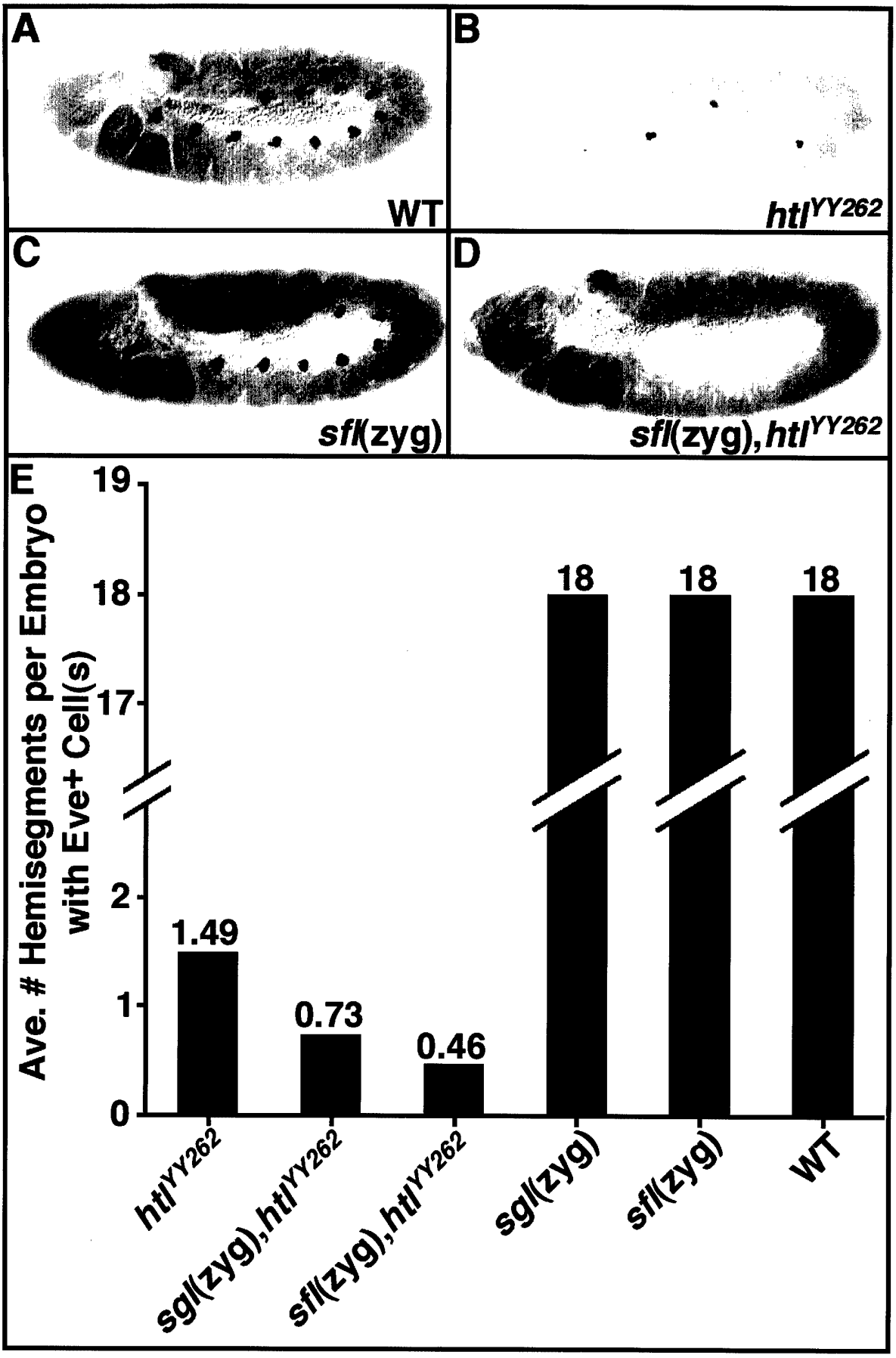
L
UAS-HtlAct sgl(mat+zyg)

Figure 1


A
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sfl(zyg)
D
sfl(zyg),htl^YY262
E
Ave. # Hemisegments per Embryo with Eve+ Cell(s)
19
18
17
2
1
0
1.49
0.73
0.46
18
18
18
htl^YY262
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sgl(zyg)
sfl(zyg)
WT

Figure 2

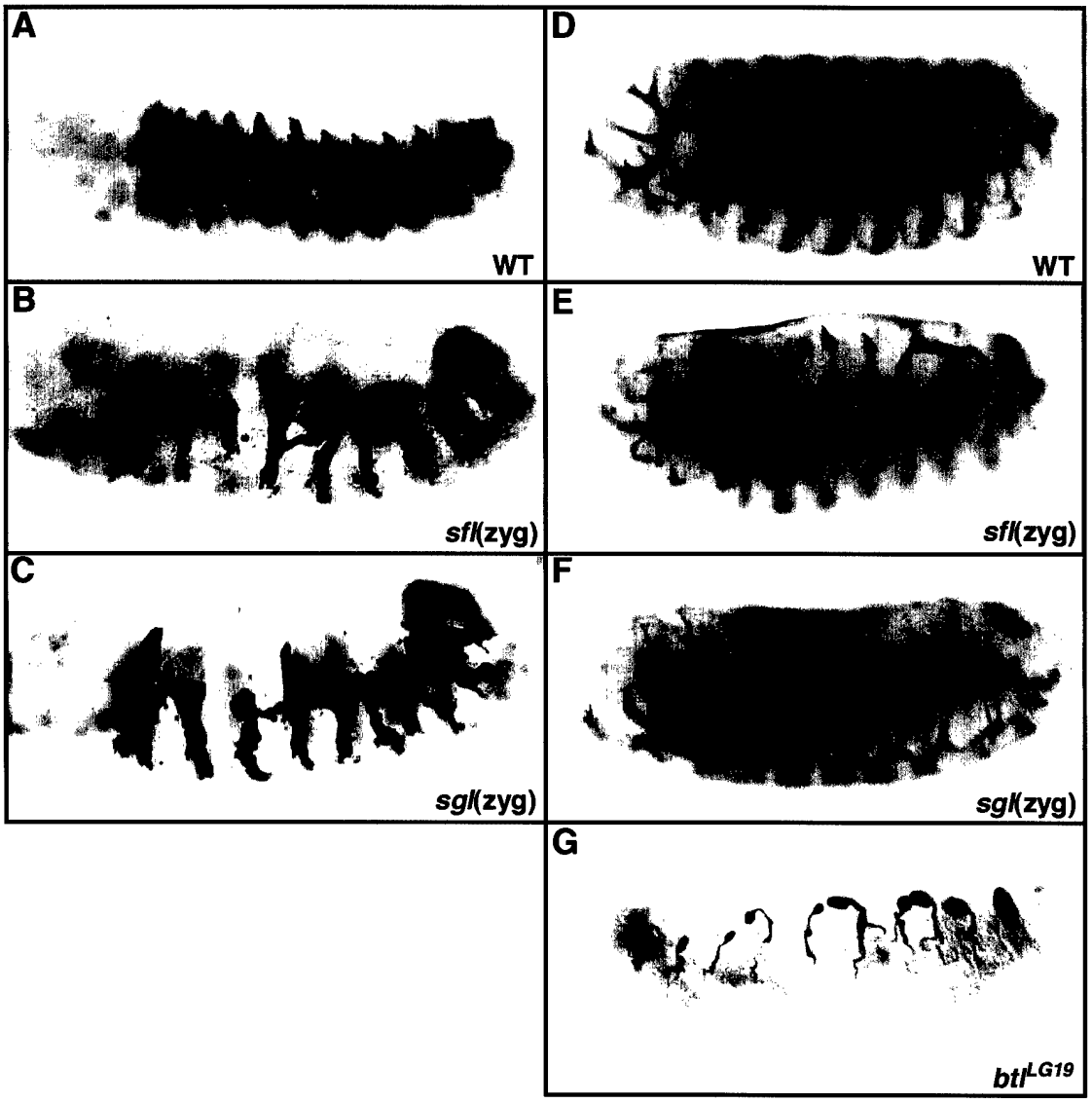
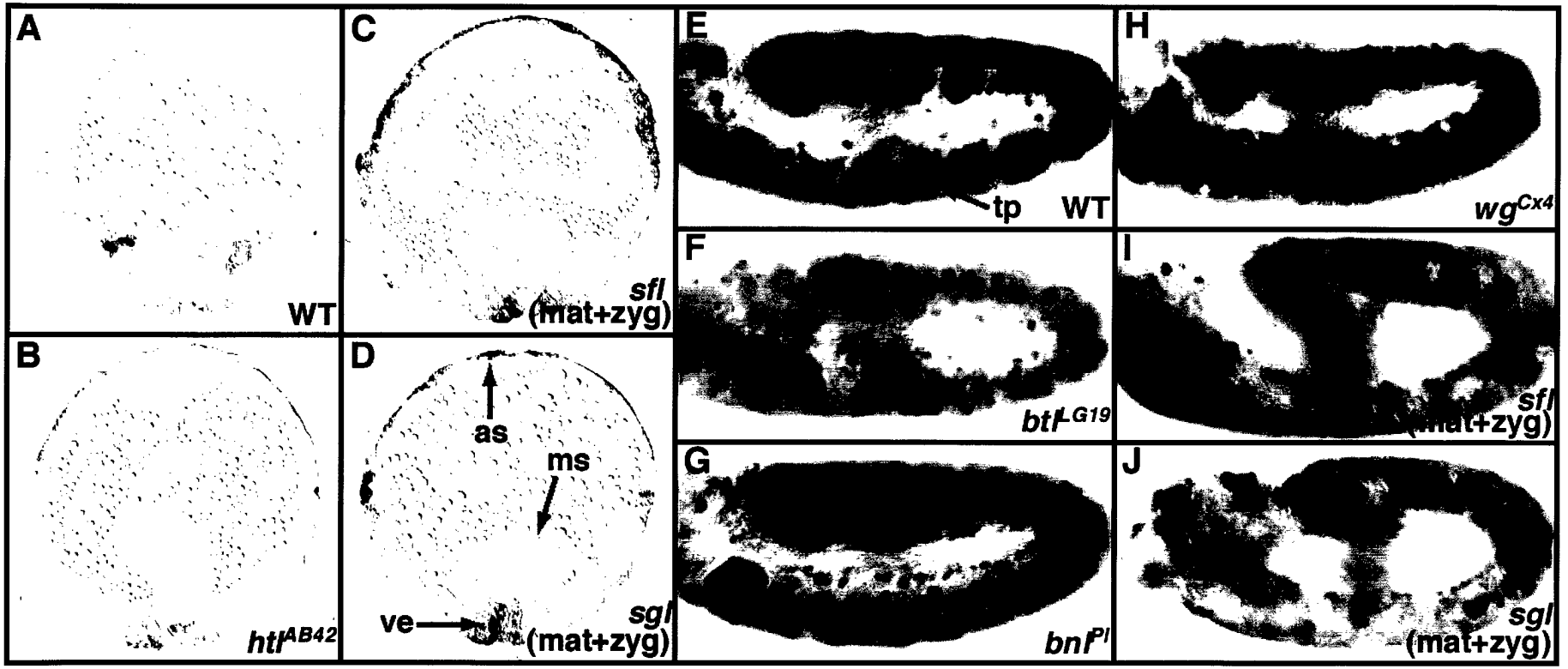


Figure 3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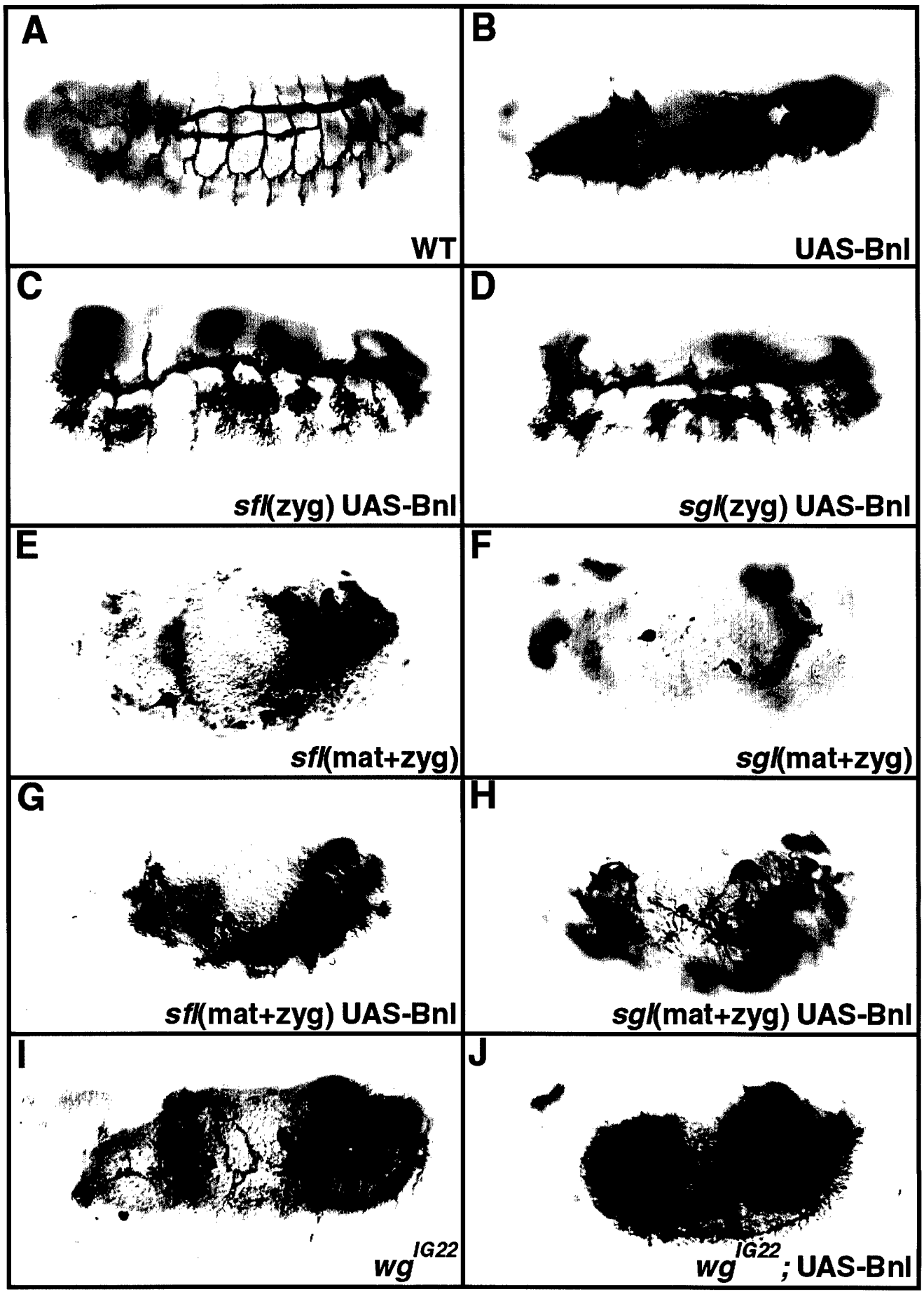
(mat+zyg)

Figure 4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wg IG22
J
wg IG22 ; UAS-Bnl

Figure 5